The Influence of Early Exposition to Environmental Microbes on Receptors of the Innate Immunity and Their Protective Effects Against Allergic Diseases

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Zusammenfassung

Die Prävalenz für eine Sensibilisierung gegenüber gewöhnlichen Allergenen, sowie die Häufigkeit allergischer Krankheiten, wie Asthma, Rhinitis, atopischer Dermatitis und Lebensmittelallergien hauptsächlich in den ersten drei Lebensjahren, ist im Laufe der letzten 50 Jahre markant gestiegen. Allergische Reaktionen sind gekennzeichnet durch ihre Produktion von IgE Antikörpern, Mastzellendegranulation und Sekretion proinflammatorischer Moleküle. Die Entstehung von Allergien ist zu einem gewissen Teil genetisch veranlagt. Es konnte jedoch bereits gezeigt werden, dass die Exposition verschiedener Umweltfaktoren, wie Mikroben oder Nahrungsmittelbestandteilen Kinder vor Allergien schützt, vor allem dann, wenn die Exposition schon während der Schwangerschaft oder früh im Leben stattfand.

Im ersten Teil dieser Dissertation untersuchten wir den Einfluss früher pre- und postnataler Expositionen diverser bäuerlichen Umweltfaktoren auf die Genexpression verschiedener Rezeptoren des angeborenen Immunsystems (Toll-like Rezeptoren und CD14). Toll-like Rezeptoren (TLRs) und CD14 erkennen konservierte Moleküle von Mikroorganismen und lösen die Aktivierung des adaptiven Immunsystems aus. Wir konnten dabei zeigen, dass bei Kindern, deren Mütter während der Schwangerschaft auf einem Bauernhof arbeiteten, verschiedene TLRs im Nabelschnurblut teilweise signifikant stärker exprimiert waren als bei Kontrollkindern. Die TLR-Genexpression nach einem Jahr war assoziiert mit Milchkonsum vom Bauernhof, besonders mit ungekochter Milch. Insgesamt weisen die Resultate darauf hin, dass sowohl die Exposition während der Schwangerschaft, als auch im ersten Lebensjahr die Expression von TLRs beeinflusst.

Im Weiteren analysierten wir den Zusammenhang zwischen TLR-Expression und der Entstehung atopischer Dermatitis in den ersten zwei Lebensjahren. Bei Kindern, die in dieser Zeitspanne an einer atopischen Dermatitis erkrankten, konnte eine signifikant tiefere Expression von TLR5 und TLR9 im Nabelschnurblut nachgewiesen werden. Schützend wirkte sich mütterlicher Kontakt während der Schwangerschaft gegenüber Bauernhofexpositionen wie z.B. Tierkontakt aus, was möglicherweise auf eine Gen-Umwelt-Interaktion schliessen lässt.

Zu guter letzt fanden wir heraus, dass eine ergänzende mütterliche Vitamin D Gabe während der Schwangerschaft in Zusammenhang mit einer erhöhten Genexpression der immunglobulin-like transcripts (ILT)3 und ILT4 steht, beides Rezeptoren für tolerogene dentritische Zellen, welche den Transkriptionsfaktor (NF)-κB und die durch ihn induzierte

Entzündungsantwort hemmen. Diese Resultate deuten auf eine frühe Induktion einer toleranzbildenden Immunantwort durch Vitamin D hin.

Der zweite Teil dieser Arbeit beschäftigt sich mit der Problematik Lebensmittelallergien zu behandeln, da die strikte Vermeidung allergieauslösender Lebensmittel immer noch die beste und einzige Methode ist um ernste anaphylaktische Reaktionen zu unterbinden. Unsere Arbeit fokussierten wir dabei auf die Desensibilisierung, eine Methode bei welcher die Menge des Allergens in steigenden Konzentrationen über eine Zeitspanne verabreicht wird und die bereits in der Behandlung von Heuschnupfen und Insektenstichallergien Anwendung findet.
Wir verwendeten attenuierte, intrazellulär lebende Bakterien (*Salmonella enterica* serovar Typhimurium) als Vektoren für die Freisetzung des Allergens im Darm. Die Allergenproduktion mittels verschiedener Bakterienstämme und Regulationsmechanismen untersuchten wir *in vitro*, während wir die Wirksamkeit unseres Konstruktes prophylaktisch und therapeutisch in einem murinen Allergensensibilisierungsmodel austesteten.
Nach kombinierter Einnahme von Mikroorganismen und Ausschüttung geringer Allergenmengen konnten abgeschwächte anaphylaktische Symptome und erhöhte antigenspezifische IgA-Werte sowie reduzierte Genexpressionen verschiedener TLRs festgestellt werden, was auf eine mögliche Eignung dieser Methode zur Prävention und/oder Behandlung von Lebensmittelallergien hindeutet. Die genauen molekularen Mechanismen bleiben allerdings weiterhin unklar und es benötigt zusätzliche Untersuchungen in diesem Bereich.

Summary

Over the last half century, the prevalence of sensitization to common allergens has increased markedly as well as the incidence of allergic diseases like asthma, rhinitis, atopic dermatitis and food allergies, mainly during the first three years of life. Allergic reactions are characterized through production of IgE antibodies, mast cell degranulation and secretion of proinflammatory molecules. The development of allergies is at least partly influenced by genetical predispositions. However, it was already shown, that the exposition to various environmental factors such as microbial or dietary components protect children against allergic diseases, mainly if the exposure occurs during pregnancy or early in life.

In the first part of this thesis we investigated various farming exposition factors during pregnancy and first year of age on the expression of receptors of the innate immunity (Toll-like receptors and CD14). Toll-like receptors (TLRs) and CD14 recognize conserved molecules of microorganisms and trigger the induction of the adaptive immune response.
We were able to demonstrate that the expression of different TLRs in cord blood of children whose mothers worked on a farm during pregnancy were partly significantly higher compared to non-farming newborns. The TLRs expression at one year of age was associated with farm milk consumption, especially unboiled farm milk. These results indicate that both, the pre- and early postnatal farming exposure influence the expression of TLRs in children.

Next, we analyzed the association between the gene expression of TLRs and CD14 and the development of atopic dermatitis in the 2 first years of life. Children, suffering from atopic dermatitis in the first two years of life have a significantly decreased expression of TLR5 and TLR9 in cord blood. Maternal farming exposure such as contact to farm animals during pregnancy has a protective effect on atopic dermatitis in the two first years of life, which may indicate a gene-environment interaction.

Finally, we found that maternal vitamin D supplementation during pregnancy was related to an increase in the gene expression of immunglobulin-like transcripts (ILT)3 and ILT4, both receptors of tolerogenic dentritic cells which inhibit the transcription nuclear factor (NF)-κB and inflammatory responses. These results may point towards an early induction of tolerogenic immune responses by vitamin D.

The second part of this thesis concerns the current major problem to treat food allergy, since strict avoidance of allergenic food is still the best method to prevent severe anaphylactic reactions. We focused our work on desensitization, a method where the amount of allergen

is administrated increasingly over a period of time and which finds appliance to treat hay fever or insect sting allergies.

We used attenuated intracellular living bacteria (*Salmonella enterica* serovar Typhimurium) as vector for allergen delivery in the gut. The allergen production by different bacterial strains and regulation mechanisms was investigated *in vitro* and the efficacy of our construct was tested prophylactically as well as therapeutically in a murine allergen sensitization model.

It could be demonstrated that the combined intake of microorganisms and the release of low amounts of allergen reduced anaphylactic symptoms combined with increased antigen-specific immunoglobulin A (IgA) titres and decreased gene expression of TLRs.

This approach may be a possible method to prevent or treat food allergies. However, the exact molecular mechanisms are still unclear and further analyses have to be done.

Introduction

Chapter overview

The human body has a tremendous capability for defence against invasion by foreign agents through its immune system. It is composed of a less specific and a more specific type of immunity, innate and adaptive immunity. The adaptive immunity is characterized through lymphocytes (B- and T-cells) producing various types of antigen receptors through gene-rearrangement. These cells show clonal expansion when stimulated with a specific antigen and exhibit immunological memory leading to a heightened state of immune reactivity after a second encounter with the same antigen. However it takes several days until the cells are able to combat invading pathogens. Therefore, the host organism has the innate immune system, which is phylogenetically conserved and present in almost all multicellular organisms. It provides the first line of defence before adaptive immunity is activated. This chapter should deliver insights into the interaction between both immune branches and the complexity behind them. Furthermore, the development of allergic disorders and their influencing factors are pointed out.

The innate immune system

Pattern recognition receptors

The innate immune recognition is mediated by a set of germline encoded non-clonal receptors which belong to several distinct protein families[1]. These receptors recognize conserved microbe-specific molecular structures, which are called pathogen-associated molecular patterns (PAMPs), and are therefore, referred as pattern recognition receptors (PRRs). PRRs can recognize PAMPs directly, through membrane-bound or cytoplasmic receptors or indirectly, whereby soluble PRRs coat or opsonize the pathogen[3]. Recognition of PAMPs by PRRs can directly activate effector mechanisms of innate immunity, such as phagocytosis, induction of synthesis of antimicrobial peptides, and induction of nitric oxide synthase in macrophages. Additionally, expression of inflammatory and effector cytokines/chemokines is induced by PAMPs, leading to recruitment of leukocytes to the sites of infection as well as stimulation and participation of the adaptive immune system in elimination of the pathogen. Finally, recognition of PAMPs by PRRs induces the expression of co-stimulatory molecules on antigen-presenting cells (APCs)[1,4].

Toll-like receptors

Toll-like receptors (TLRs) are one group of PRRs which recognize highly conserved microbial structures and thereby initiate an immune response. The discovery of the TLR familiy began with the isolation of *Drosophila* Toll, which had initially been identified as having a role in embryonic dorso-ventral patterning[5] but is also an essential factor in the insect innate immune response against fungal infection[6]. Ten different homologues of Toll were identified through database searches in humans (TLR1-TLR10) and twelve TLRs in mice (TLR1-9, TLR11-13)[2,7]. Each of them recognizes specific components found on bacteria and viruses (Table 1). They are structurally similar transmembrane PRRs with a cytoplasmic and an extracellular portion (Fig. 1). The cytoplasmic domain shows high similarity to that of the mammalian IL-1 receptor and is called Toll/IL-1 receptor (TIR) domain. The TIR domain consists of three conserved boxes (Box 1-3), which are crucial for signalling. By contrast, the extracellular domain of the IL-1 receptor and TLRs differs markedly: The extracellular domain of TLRs consist of leucine-rich repeats (LRRs) whereas the IL-1 receptors possess an Ig-like domain[2]. TLRs are differentially expressed on a wide range of immune cells including T- and B-lymphocytes, dendritic cells, macrophages and neutrophiles, as well as on non-immune cells, such as fibroblast cells, epithelial cells and keratinocytes[8,9]. TLR 1, 2, 4, 5, and 6 are located on the cell surface and are mainly responsible for the detection of bacterial products, whereas the subset of TLRs that sense nucleic-acid-like structures (TLRs 3, 7, 8 and 9) are expressed intracellularly on endosomal membranes[2,10].

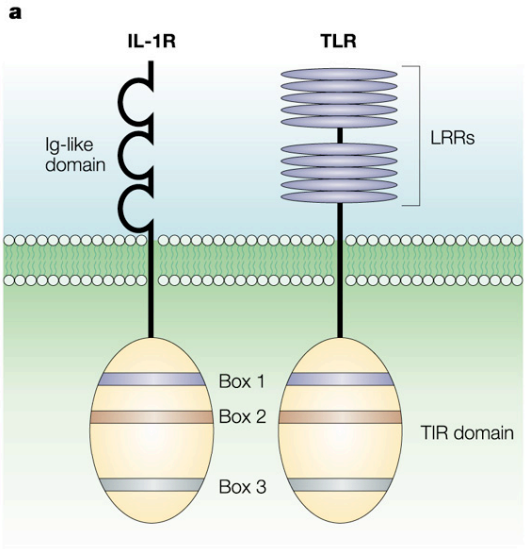


Figure 1.: Schematic structure of Toll-like receptor (TLR) and IL-1 receptor (IL-1R). Both receptors have a conserved intracellular Toll/IL-1 receptor (TIR) domain, characterized by the presence of three highly homologues regions (Box 1-3). TLRs have a leucine-rich, extracellular domain, whereas IL-1Rs have immunoglobulin (Ig)- like domain. Adapted from[2].

Table 1 | Pattern recognition receptors in humans

Receptor	Ligand	Origin of ligand
TLR1	Triacyl lipopeptides	Bacteria and mycobacteria
	Soluble factors	*Neisseria meningitidis*
TLR2	Lipoprotein/lipopeptides	Various pathogens
	Peptidoglycan	Gram-positive bacteria
	Lipoteichoic acid	Gram-positive bacteria
	Lipoarabinomannan	Mycobacteria
	Phenol-soluble modulin	*Staphylococcus epidermidis*
	Glycoinositolphospholipids	*Trypanosoma cruzi*
	Glycolipids	*Treponema maltophilum*
	Porins	*Neisseria*
	Atypical lipopolysaccharide	*Leptospira interrogans*
	Atypical lipopolysaccharide	*Porphyromonas gingivalis*
	Zymosan	Fungi
	Heat-shock protein 70	Host
TLR3	Double-stranded RNA	Viruses
TLR4	Lipopolysaccharide	Gram-negative bacteria
	Taxol	Plants
	Fusion protein	Respiratory syncytial virus
	Envelope protein	Mouse mammary-tumour virus
	Heat-shock protein 60	*Chlamydia pneumoniae*
	Heat-shock protein 70	Host
	Type III repeat extra domain A of fibronectin	Host
	Oligosaccharides of hyaluronic acid	Host
	Polysaccharide fragments of heparan sulphate	Host
	Fibrinogen	Host
TLR5	Flagellin	Bacteria
TLR6	Diacyl lipopeptides	*Mycoplasma*
	Lipoteichoic acid	Gram-positive bacteria
	Zymosan	Fungi
TLR7	Imidazoquinoline	Synthetic compounds
	Loxoribine	Synthetic compounds
	Bropirimine	Synthetic compounds
	Single-stranded RNA	Viruses
TLR8	Imidazoquinoline	Synthetic compounds
	Single-stranded RNA	Viruses
TLR9	CpG-containing DNA	Bacteria and viruses
TLR10	Not determined	Not determined
NOD-1	Peptidoglycan	Gram-negative and few gram-positive bacteria
NOD-2	Muramyl dipeptides	Gram-positive/negative bacteria

Adapted from[2]

TLR1, TLR2, TLR6

TLR2 recognizes the largest number of ligands of the mammalian TLRs. The list includes lipoproteins from a variety of pathogens, lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, glycosylphophatidylinositol anchors from *Trypanosoma cruzi*, a phenol-soluble modulin from *Staphylococcus epidermis*, zymosan from fungi and glycolipids from *Treponema maltophilum*[11,12]. The mechanisms by which TLR2 recognizes an increased repertoire of microbial components is now known by the fact that TLR2 forms heterodimers with either TLR1 or TLR6[13,14].

TLR4 and CD14

TLR4 was the first human TLR identified to play an essential role in recognition of lipopolysaccharide (LPS), the major component of the outer membrane of Gram-negative bacteria. However, the response to LPS requires several additional molecules. LPS binds to LPS-binding protein (LBP), an acute-phase protein in serum produced by hepatocytes and this complex associates with CD14. CD14 is viewed also as pattern recognition receptor[15] which is able to interact not only with LPS but with various bacterial products, including lipoarabinomannan from *Mycobacterium tuberculosis*[15], peptidoglycan from *Staphylococcus aureus*[16], mannuronic acid polymers from *Pseudomonas aeruginosa*[17], rhamnose-glucose polymers from *Streptococcus mutans*[18], and chitosans from arthropods[19]. CD14 exists in a membrane bound (mCD14) and a soluble (sCD14) form. mCD14 is a glycosyl-phosphatidylinositol (GPI)-linked glycoprotein, expressed on monocytes/macrophages and neutrophiles[20], whereas sCD14 is found in human plasma[21] and has the ability to stimulate CD14-negative cells, such as endothelial cells, astrocytes, and epithelial cells[22].

Another protein involved in the response to LPS is MD-2, that binds both the extracellular domain of TLR4 and the hydrophobic portion of LPS[3]. MD-2 has been shown to be essential for surface expression of TLR4, and therefore mediated the responsiveness to LPS[23,24]. An additional molecule involved in the recognition of LPS is RP105, preferentially expressed in B-cells. RP105 bears leucine-rich repeats that are structurally related to TLRs in the extracellular domain.

TLR5

TLR5 recognizes a conserved domain of flagellin, a subunit of the flagellar structure, present in many different bacteria like *Salmonella* or *Legionella*.

This receptor is expressed on the basolateral but not on the apical side of the intestinal epithelial cells[25] as well as on lung epithelial cells[26] and has an important role in microbial recognition at the mucosal surface.

TLR3, 7, 8, 9

TLR3, 7, 8 and 9 are all located in the endosomal membrane[2] and are involved in the recognition of nucleic-acid-like structures. TLR7 and 8 are essential for the recognition of guanosine- or uridine-rich single-stranded RNA (ssRNA) from viruses[27], whereas TLR9 recognizes unmethlyated Cytosin-phosphatidyl-Guanosin (CpG) motifs present in bacterial and viral DNA[28]. TLR3 is involved in recognition of double-stranded RNA (dsRNA)[29], which is produced by most viruses during their replication.

TLR10

The specific ligands for TLR10 are currently unknown. However, protein complexes with TLR10 have been found as homodimer and heterodimers, associated with TLR1 or TLR2. The expression seems to be restricted to B-cells and plasmacytoid dendritic cells (PDCs), similar to TLR7 and TLR9, indicating a related role of TLR7, TLR9 and TLR10 in terms of biological functions[30].

Nucleotide-binding oligomerization domain (NOD)-like receptors

Nod-like receptors (NLRs) are a family of intracellular sensors that play key roles in innate immunity and inflammation[31]. These proteins have been identified at first on the basis of their homology with the plant R proteins, which is a large family of proteins that mediate antipathogen responses in plants[32]. The human NLR family has 23 members and there are at least 34 NLR genes in mice[3,33]. The structure of NLR proteins is tripartite. First, a variable N-terminal protein–protein interaction domain, defined by the caspase recruitment domain (CARD), pyrin domain (PYD), or the baculovirus inhibitor domain (BIR), second, a centrally located NOD domain that facilitates self-oligomerization during activation and third, a C-terminal leucine-rich repeat (LRR) responsible for binding and detecting PAMPs[34].

NOD1 and NOD2

NOD1 and NOD2 can be viewed as the ‘germinal’ and the best characterized members of the NLR family[3,33]. NOD1 is ubiquitously expressed and recognizes a specific core motif of peptidoglycans of Gram-negative, but only a few strains of Gram-positive bacteria[35,36]. In comparison, NOD2 expression is restricted to monocytes, macrophages, dendritic cells, and

intestinal Paneth cells and peptidoglycan muramyl dipeptide of all Gram-positive and Gram-negative bacteria are detected by this receptor[37].

TLR signalling pathway

After ligand binding, TLRs dimerize and undergo a conformational change in the cytoplasmic TIR domains, creating a new platform on which to build a signalling complex. Via TIR-TIR interactions different adaptor proteins are recruited, including myeloid differentiation primary response 88 protein (MyD88), TIR-containing adaptor protein/MyD88-adaptor-like (TIRAP/MAL), TIR-containing adaptor inducing interferon-β (IFN-β)/TIR-domain-containing adaptor molecule 1 (TRIF/TICAM1) and TIR-domain-containing adaptor molecule/TRIF-related adaptor molecule 2 (TRAM/TICAM2) and sterile α- and armadillo-motif containing protein (SARM)[7,9]. Similar to the TLRs, the adaptors are conserved across many species[38,39]. Subsequent recruitment of downstream signalling molecules leads to activation of nuclear factor-κB (NF-κB) transcription factors, as well as the mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38, and c-Jun N-terminal kinase (JNK)[40].

The resulting signalling pathways may generally be grouped into MyD88-dependent and MyD88-independent/TRIF-dependent pathways.

MyD88-dependent pathway

MyD88-dependent signalling pathway is shared by all members of the TLR family except TLR3 and results in the induction of a core set of responses. MyD88 was first shown to be involved in signalling by the type 1 IL-1 receptor (IL-1R1)[41]. MyD88 comprises a C-terminal TIR domain and a N-terminal death domain (DD), separated by a small intermediate domain (ID)[42]. After TLR stimulation MyD88 is recruited to the cytoplasmic portion of the TLR and interacts with the death domains of members of the IRAK (IL-1 receptor-associated kinase) family of protein kinases, including IRAK1, IRAK2, IRAK4 and IRAK-M. IRAK4 is initially activated and in turn phosphorylates and activates IRAK1 which subsequently interacts with TNFR-associated factor-6 (TRAF6), leading to oligomerization and activation of TRAF6[7,9,41,43]. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1 (transforming-growth-factor-activated kinase), TAB1 (TAK1-binding protein 1) and TAB2 at the plasma membrane, which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 (ubiquitin-conjugating enzyme 13) and UEV1A (ubiquitin-conjugating enzyme E2 variant 1). Thereby TRAF6 is ubiquitinylated and induces

the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-B (IB)-kinase complex). This complex consists of IKK-α, IKK-β and IKK-γ/NEMO (NF-κB essential modifier) and phosphorylates IκBs (NF-κB inhibitors), resulting in its ubiquitination and subsequent proteosome-mediated degradation. This releases NF-κB, which moves to the nucleus and regulates its target genes, including those that encode proinflammatory cytokines[7,9,41,43].

Beside the participation of MyD88 in the activation of mitogen-activated protein kinase (MAPK) pathway[7,9,41,43] it is also involved in the activation of several transcription factors of the interferon-regulatory factor (IRF) family, namely, IRF1, IRF7 and IRF5[7]. IRF5 is involved in the induction of proinflammatory cytokines and type I interferones (IFNs) by all TLRs tested[44]. Activated TLR7 or TLR9 of plasmacytoid dentritic cells (pDCs) lead to activation of a MyD88 which directly interacts with IRF7, an essential transcription factor for IFN-α/β induction[45,46]. A simplified illustration of the MyD88-dependent pathway is depicted in Figure 2.

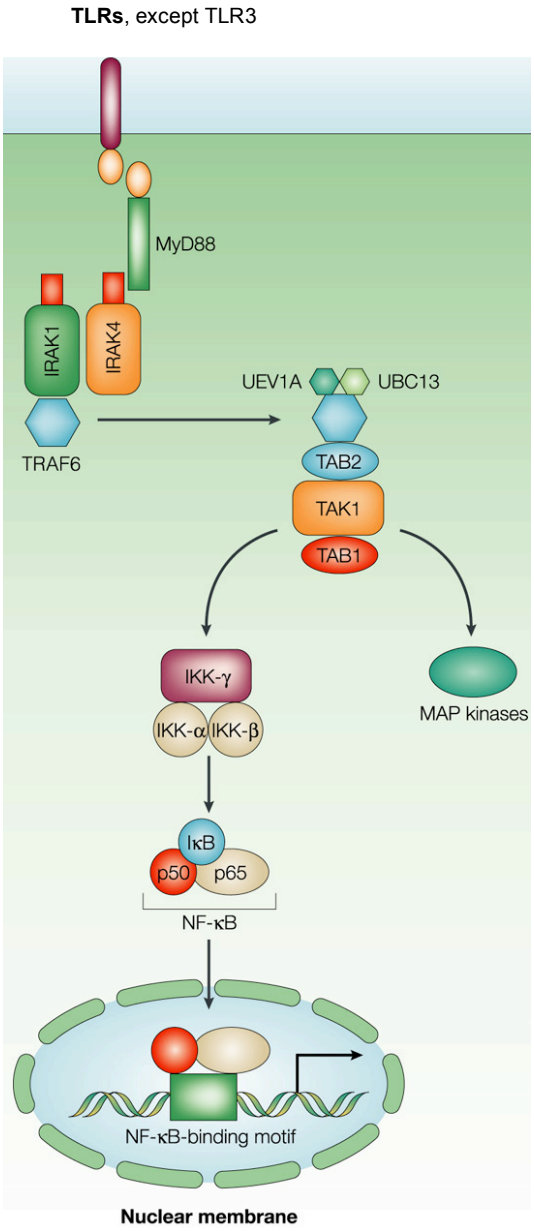
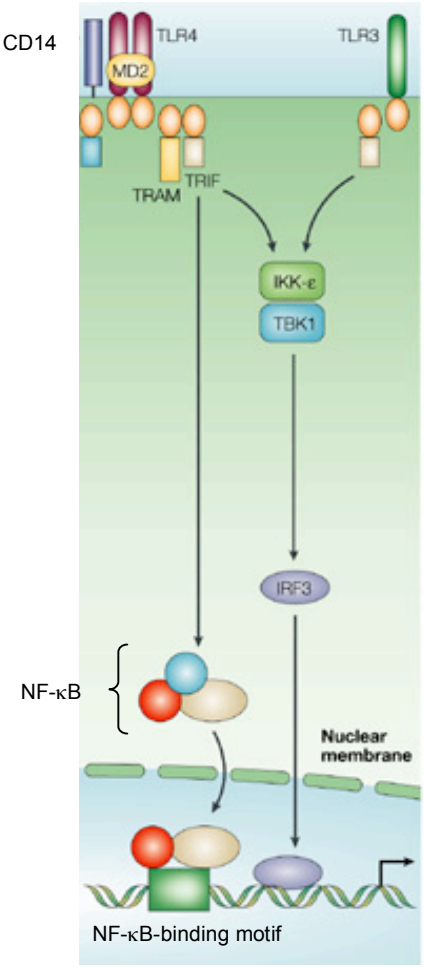


Figure 2. Stimulation of TLRs triggers the association of MyD88, which in turn recruits IRAK4, thereby allowing the association of IRAK1. TRAF6 is also recruited to the receptor complex, by associating with phosphorylated IRAK1. Phosphorylated IRAK1 and TRAF6 then dissociate from the receptor and form a complex with TAK1, TAB1 and TAB2 at the plasma membrane (not shown), which induces the phosphorylation of TAB2 and TAK1. IRAK1 is degraded at the plasma membrane, and the remaining complex (consisting of TRAF6, TAK1, TAB1 and TAB2) translocates to the cytosol, where it associates with the ubiquitin ligases UBC13 and UEV1A. This leads to the ubiquitylation of TRAF6, which induces the activation of TAK1. TAK1, in turn, phosphorylates both mitogen-activated protein (MAP) kinases and the IKK complex (inhibitor of nuclear factor-κB (IκB)-kinase complex), which consists of IKK-α, IKK-β and IKK-γ. The IKK complex then phosphorylates IκB, which leads to its ubiquitylation and subsequent degradation. This allows NF-κB to translocate to the nucleus and induce the expression of its target genes. Adapted from [2].

MyD88-independent/TRIF-dependent pathway



As described above, the adaptor protein MyD88 is not required for NF-κB activation by TLR3. The TIR-domain-containing adaptor molecule (TRIF) was shown to be essential for TLR3 as well as TLR4 mediated activation of interferon (IFN)-regulatory factor 3 (IRF3) in a MyD88 independent manner[47]. TRIF interacts with receptor-interacting protein 1 (RIP1) and also with TRAF3, which bridges to TBK1 and IKK-related proteins – IKKε (also known as inducible IKK, IKK*i*)[48]. TLR4 mediated MyD88-independent IRF3 activation and interferon β (IFN-β) production needs in addition to TRIF also TRAM as adaptor molecule[49]. The activation of IRF3 by signalling through TLR4 is less rapid and potent compared to TLR3 signalling, leading to decreased IFN-β production[2] (Fig. 3).

Figure 3. MyD88-independent signalling pathway. The TIR domain-containing adaptor molecule TRIF is recruited to TLR3 and 4 after ligand binding. TLR4 requires additionally the adaptor protein TRAM. Downstream of TRIF, IRF3 is activated through the IKK/TBK1 complex. Adapted from[2]

Other molecules involved in TLR signalling

Negative regulators of TLR signalling

Inflammatory cytokine production through activation of TLR signalling has to be controlled, otherwise severe systemic disorders are elicited, such as endotoxic shock reaction, induced by TLR4/lipopolysaccarid (LPS) binding. Therefore, the organisms have evolved mechanisms which modulate the TLR-mediated response. Some of these negative regulators are shown in Figure 4 and discussed briefly here.

MyD88 short (MyD88s)

MyD88s is an alternative spliced variant of MyD88, the most crucial adaptor in TLR signalling, that lacks the interdomain. While MyD88 is ubiquitously expressed, MyD88s was

only detected in the spleen and, less strongly, in the brain. It was shown that MyD88s is not able to bind IRAK4 because of the missing intermediary domain and does not induce IRAK1 phosphorylation, indicating that MyD88s is a member of the negative-feedback regulatory system[2,50,51].

IRAK-M and IRAK-2

IRAK-2 and IRAK-M are members of the IRAK family that lack active serine/threonine kinases. IRAK-2 is expressed ubiquitously, whereas the expression of IRAK-M is restricted to monocytes and macrophages and upregulated following stimulation with TLR ligands. IRAK-M negatively regulates TLR-signalling pathways by preventing the dissociation of the IRAK1-IRAK4 complex from MyD88. Consequently the IRAK1-TRAF6 complex does not form[50,51]. Four isoforms of murine IRAK-2 are known (IRAK2a-IRAK2d) generated by alternative splicing at the 5`end of the gene. Overexpression of IRAK2c and IRAK2d in fibroblasts inhibits LPS-induced NF-κB activation in a dose-dependant manner[52]. Furthermore treatment of murine macrophages with LPS induces the expression of IRAK2c indicating a possible negative feedback effect on the signalling pathway[50,53].

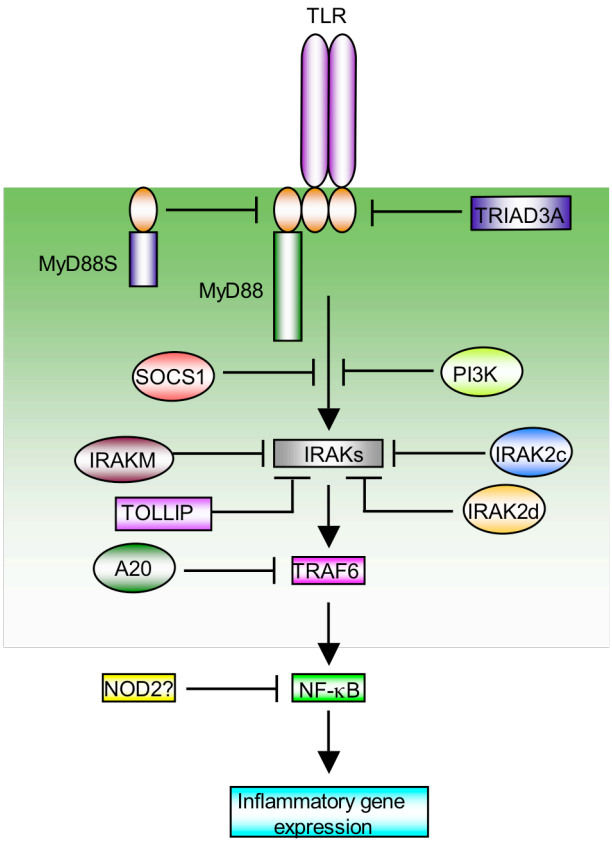


Figure 4. Intracellular Toll-like receptor regulators. TLR signalling pathways are tightly regulated by endogenous regulators at multiple levels. TRIAD3A promotes ubiquitylation and degradation of certain TLRs, whereas MyD88s (the short form of MyD88) antagonizes MyD88 functions. Inhibitory proteins such as SOCS1 (suppressor of cytokine signalling 1), IRAKM (Toll-interacting protein), IRAK2c and IRAK2d selectively suppress IRAK function by targeting various stages of the TLR signalling pathways. Phosphatidylinositol 3-kinase (PIK3K) negatively regulates some TLR responses through an as-yet-unknown mechanism. A20 deubiquitylates TRAF6 (tumour-necrosis factor-receptor-associated factor 6) and affects both MyD88-dependent and MyD88-independent pathways. NOD2 might inhibit TLR2 signalling by suppressing nuclear factor-κB (NF-κB) activity. Adapted from Liew F. et al.; Nature (2005), 5. 446-458.

SOCS1

SOCS1 is a member of the suppressor of cytokine signalling (SOCS) family of proteins, which are induced by cytokines and are involved in a negative feedback loop of cytokine signalling. Eight members of the SOCS family are identified (SOCS1-7 and Cytokine-inducing SH2-containing protein (CIS)) and all of them are characterized through an amino-terminal region of variable length, a central SH2 domain and a carboxy-terminal SOCS box.

SOCS1 and SOCS3 have additional a kinase inhibitory domain (KIR) and both act in a similar manner. SOCS1 is one of the best characterized proteins of the SOCS family, but the precise mechanism by which SOCS1 inhibits TLR signalling is still unclear. SOCS1 expression is induced by cytokines that signal through JAK/STAT molecules but also after stimulation with LPS and CpG-containing DNA. *In vivo* studies have shown that SOCS1-deficient mice are hypersensitive to LPS-induced endotoxic shock, leading to increased production of inflammatory cytokines. Furthermore, LPS tolerance is not induced in SOCS1 deficient mice and LPS-induced IκB and p38 phosphorylation are upregulated in SOCS1 deficient macrophages[53,54]. Children growing up on a farm, in an environment rich in microbes show an upregulation of SOCS1 expression compared to their peers not living on a farm[55]. Furthermore higher expression of SOCS1 gene is associated with reduced IgE and IFN-γ production[55]. However, a recent epidemiological study show that SOCS1 correlates with Th2 development and a higher risk for allergic sensitization[56].

ST2

ST2, also known as T1, Fit-1 or DER4, is a TIR-containing orphan receptor, which is involved in the negative regulation of TLR signalling. Two main forms of ST2 exist: ST2L and sST2 which are generated by alternative splicing of pre-mRNA. ST2L is a type I transmembrane protein characterized through three extracellular immunoglobulin-like domains and an intracellular TIR domain. The extracellular domain of sST2 is similar to the one of ST2L but the transmembrane and intracellular Toll-interleukin-1 receptor domains lack. sST2 is present in haematopoietic and non-haematopoietic cells, whereas ST2L is mainly expressed by cells of the major haematopoietic organs[57-59] preferentially in murine and human Th2 but not Th1 cells[60,61]. Therefore, ST2L can be used as a specific marker for Th2 cells in *in vitro* experiments[62]. On the other hand, sST2 is present in normal human serum and elevated in asthmatic disease[63]. However, therapeutic experiments indicate that sST2 negatively regulates TH2 cell-mediated immunological responses, in contrast to ST2L[64,65]. Furthermore, gene expression analyses have shown that sST2 is at lower levels expressed in children exposed to microbial components and this, in turn, is associated with reduced T helper cell activation, lower IgE levels in serums, and hay fever[66]. It is currently unknown how sST2 influences the TLR signalling cascade negatively but *in vitro* and *in vivo* analysis have shown that sST2 is able to downregulate mRNA expression of TLR4 and TLR1 in LPS-activated macrophages[67] and suppresses the activation of NF-κB as well as the production of TH2 cytokines in IL-33 signalling[68].

Single immunoglobulin IL-1R-related molecule (SIGIRR)

SIGIRR, also known as Toll–IL-1 receptor 8 (TIR8), is an orphan receptor like ST2L and characterized by a single extra-cellular immunoglobulin domain and a cytoplasmic TIR domain[69]. It has been identified as a negative regulator for IL-1R[70], TLR4[71], and TLR9 signalling[70,72]. Mice, lacking SIGIRR, develop more severe inflammatory responses to IL-1 and show increased susceptibility to endotoxin shock[51,53]. The detailed molecular mechanisms by which SIGIRR suppresses TLR function are currently unknown but *in vitro* analysis has shown that SIGIRR can interact with TLR4, IRAK and TRAF6[51,73].

TNF-related apoptosis-inducing ligand receptor (TRAILR)

TRAILR is a receptor, which belongs to the TNF superfamily and binds TRAIL, also referred as Apo-2L. TRAILR-deficient macrophages stimulated by ligands for TLR2, TLR3 and TLR4, express high levels of TRAIL and show enhanced cytokine production. The inhibitory effect of TLR signalling seems to function by stabilizing the IκB subunit IκBα and therefore decreasing the nuclear translocation of NF-κB[51].

Phosphoinositide 3-kinase (PI3K)

PI3K is a heterodimer that consists of a p85 regulatory subunit and a p110 catalytic chain, both necessary for PI3K activation. The activation of PI3K occurs through binding of microbial stimuli such as LPS, CpG DNA or CpG ODN (oligodeoxynucleotide) and plays an important role in regulating immune cell defensive mechanisms, including cytokine production, phagocytosis, and apoptosis. PI3K seems to have a positive and a negative influence on TLR signalling depending on the immune cell type and the TLR ligand used[74]. Different PI3K catalytic subunit isoforms exist which partly are involved in allergic airway inflammation and hyperresponsiveness. Therefore PI3K is dealt as a potential target for asthma treatment[75].

A20

A20 is a 90-kDa zinc-finger protein which is activated by a variety of stimuli including synthetic RNA (poly I:C), LPS, and TNF-α[76]. A20 deficient mice injected with TNF-α show severe inflammation and elevated sensitivity to endotoxin shock, due to persistent activation of NF-κB[77]. Furthermore, A20 was shown to cleave the essential ubiquitin chain for downstream signalling of TRAF6[78], indicating that A20 is the only negative regulator that control MyD88-dependent as well as MyD88-independent TLR-signalling pathways[51,78].

SHP2

The tyrosine phosphatase SHP2 (SRC homology 2 (SH2)-domain-containing protein) has recently been shown to dephosphorylate STAT1[79] and negatively regulates the TRIF-dependent, but not MyD88-dependent interferon and proinflammatory cytokine production[80].

TRIAD3A

TRIAD3A is the most abundant alternatively spliced form of the TRIAD3 family and is a RING-finger E3 ligase. It was shown that TRIAD3A binds the cytoplasmic TIR domains of TLR4 and TLR9 and promotes their ubiquitylation and degradation, leading to a negative regulation of TLR mediated immune response[81].

NOD2

As described before, nucleotide oligomerization domain (NOD)-like receptor 2 is a pattern recognition receptor, which recognizes the bacterial product muramyl dipeptide (MDP), a derivative of bacterial peptidoglycan[82]. MDP was found to suppress TLR2-ligand-induced T-helper cell type 1 (Th1-cell) responses in wild-type, but not in NOD2-deficient mice, indicating that NOD2 is a negative regulator of TLR2 signalling[51]. However, results from human studies could not confirm this observation but suggest a synergistic effect between NOD2 and TLR2 in a dose dependent manner[83].

TOLLIP

The Toll-interacting protein (TOLLIP) serves as a negative regulator in innate immune response. TOLLIP interact with several members of the TIR superfamily, including TLR2 and TLR4[84] and is able to inhibit TLR2- and TLR4-mediated NF-κB activation, if it is over-expressed[84,85]. The exact molecular mechanism of TOLLIP is not known. It has been shown that IRAK1 interacts with TOLLIP leading to phosphorylation of TOLLIP[86]. It is not clarified, if this phosphorylation step results in ubiquitinylation of IRAK1 and its subsequent degradation which might lead to the release of TOLLIP and its negative regulation[51]. In opposite to many other inflammation suppressors including IRAK-M, MyD88s, TOLLIP is constitutively expressed and also present in unstimulated cells together with other binding partners, suggesting that the function of TOLLIP is of more multifaceted nature[87].

Innate immune recognition and activation of adaptive immunity

Unlike innate immunity, the adaptive immunity has a high antigenic specificity, capable to recognize a tremendous number of diverse structures on foreign antigens, exhibits immunological memory and distinguishes self from nonself. The signals to activate this branch of the immune system are provided by antigen presenting cells (e.g. macrophages, dendritic cells, microglia, B-cells) after recognition of bacterial and viral components by receptors of the innate immunity (e.g. TLRs). The antigen binding leads to antigen uptake and processing. The degraded peptides are bound to MHC (major histocompatibility complex) class II molecules and presented to $CD4^+$ T-lymphocytes leading to activation and expansion of $CD4^+$ T-cells[88].

MHC molecules

There are three classes of MHC molecules (MHCI, MHCII, MHCIII) encoded by different gene regions. The genes for MHCII are organized in the DP, DQ, and DR regions. MHCI and MHCII are membrane-bound glycoproteins that contain extracellular domains, a transmembrane segment, and a cytoplasmic anchor segment. Both are involved in antigen processing and presentation. In contrast, MHCIII genes encode various proteins that have immune functions, including tumor necrosis factor (TNF) or the complement components C4, C2, and factor B. MHCII molecules contain a 33 kDa α-chain and a 28 kDa β-chain, associated by noncovalent interactions[4]. The MHCII production is regulated at the level of transcription by CIITA (class II transactivator), which interact synergistically with the MHCII transcription factors regulatory factor X (RFX) 5, RFX associated ankyrin-containing protein (RFXANK), RFX-associated protein (RFXAP), nuclear factor binding to the Y box (NF-YB and NF-YB), and X2-box binding factor cyclic-AMP-responsive-element-binding (CREB) protein, referred as MHCII enhanceosome[89]. The transcription factors are more or less ubiquitously expressed whereas CIITA is regulated precisely in different cell types through cytokines or during particular stages of differentiation and influences therefore MHCII expression directly[90,91]. Peptides bound on class II molecules contain in general 13 to 18 amino acid residues and have often internal conserved sequence motifs, but in contrast to MHCI peptides, conserved anchor residues are missing[4]. Antigenic peptides displayed in the groove of MHCII are recognized by $CD4^+$ T-cells by interaction with the T-cell receptor (TCR).

T-cell subtypes

Antigen recognition by naïve $CD4^+$ T-cells leads, depending on the cytokines in the microenvironment, to activation, proliferation and differentiation in at least 4 types of effector T-cells (Th1, Th2, Th9, Th17). These Th-cell supopulations differ in their effector functions, based on the expression of transcription factors, cytokine production, chemokine responses and mobilization of other cell types (Fig. 5).

Th1/Th2

Th1-cells express the transcription factor Tbet, produce IFN-γ, TNF-β and Interleukin (IL) 2 and mediate protection against intracellular pathogens, whereas Th2-cells express the transcription factor GATA-3, secrete cytokines like IL-4, IL-5, IL-9, IL-13, IL-25, IL-31, and IL-33 and promote the elimination of parasitic helminth infections and are involved in allergic reactions[92,93]. GATA-3 inhibits the production of IFN-γ and increases the transactivation of the IL-4 promotor as well as the direct regulation of IL-5 and IL-13 expression. In contrast, Tbet develops IFN-γ production and simultaneously represses the Th2 cytokines IL-4 and IL-5[94]. Th1- and Th2-cell differentiation is induced by IL-12 and IL-4, respectively *in vitro*. However, many Th1-cell responses *in vivo* are partially or completely independent of IL-12, including those that cause diabetes in non-obese diabetic mice[95] and those that are generated in response of soluble extract from *Toxpoplasma gondii*[96] or viruses, such as lymphocytic choriomeningitis virus and mouse hepatitis virus[97-99], suggesting that IL-12 selects cells that are already committed to the Th1-cell lineage and enhances their effector function instead of Th1-cell differentiation induction[100]. Similar to Th1, Th2-cell responses *in vivo* are mostly independent of IL-4, following infection with helminths[101]. It has been argued that IL-4 might mainly function to amplify Th2-cell responses and might be necessary when other signals that drive Th2-cell differentiation are limiting[100]. The exact signals leading to T-cell differentiation are unknown, but there is evidence which suggests that the Notch pathway can provide these signals[100]. Notch is a heterodimeric cell-surface receptor, expressed by $CD4^+$ T-cells and involved in a broad range of differentiation and development processes[102]. In mammals two distinct families of Notch ligands are known, referred as Delta-like ligands (DLL) and the Jagged ligands. DLL proteins are induced through bacterial exposure (*Acinetobacter lwofii* and *Lactococcus lactis*) and exposure to TLRs ligands (LPS and CpG-containing DNA) as well as infection with respiratory syncytial virus. Induction of Th1-cell differentiation by APCs correlates with the expression of DLL proteins, suggesting that the Notch pathway supports Th1-cell differentiation[100]. The inhibition of Notch pathway by γ-secretase blocks IL-12-induced Th1-cell differentiation *in vitro* and *in vivo*[103]. However, experiments with Notch knockout mice have not confirmed if Notch alone is required for Th1-

cell differentiation or if other proteins are also involved. Recent data from Fang et al[104] have shown that specific blocking of Notch signalling in peripheral naïve $CD4^+$ T-cells results in a loss of IL-4 production and integration of an activated Notch1 allele into naïve $CD4^+$ T-cells leads to robust IL-4 induction, indicating that IL-4 production is regulated by Notch in a $CD4^+$ T-cell-intrinsic manner. Furthermore, it has also been shown that Notch induces GATA3 expression in a STAT6-independent manner, resulting in the stabilization of the Th2-cell phenotype[100,104]. GATA3 expression might be a key element that determines whether Notch induce Th1- or Th2 cell differentiation, but the molecular mechanisms that favour or prevent the expression of this crucial Th2-cell transcription factor are currently unclear[104].

As written above, Th1- and Th2-cells determine different biological functions and the progression of some diseases. The Th1-cell population is responsible for delayed-type hypersensitivity, production of IgG antibodies, excessive inflammation, autoimmune disease and tissue injury, whereas the Th2-cell subset activates eosinophils, supports immunglobulin-class switch to IgG1 and IgE and is involved in the development of airway inflammation, asthma and atopy.

Th9/Th17

The recent identification of the IL-17 and IL-9 producing T-helper (Th17 and Th9, respectively) cells has added complexity to the $CD4^+$ effector T cell paradigm. Th17 cells are characterized by the production of IL-17, IL-17F and IL-22. IL-17 and IL-17F induce the production of proinfammatory cytokines, chemokines and metalloproteinases from various tissues and cell types, resulting in the recruitment of neutrophils to tissues[105]. Th17-cell differentiation and expansion does not require IL-17 in contrast to Th1- and Th2-cell differentiation, which is driven by their effector cytokines IFN-γ and IL4, respectively. Th17 differentiation is instead induced by IL-6 and TGF-β[106]. In absence of IL-6, IL-21 produced by natural killer cells and natural killer T-cells, acts as an alternative inductor of Th17-cell differentiation[107]. The Th17 lineage-specific transcription factor is presumably STAT3 because deletion of STAT3 prevents Th17 development[108]. In addition, the orphan nuclear receptor ROR-γt was also identified as lineage specific transcription factor for Th17 cells[109]. A Th17 response is induced by extracellular Gram-negative (*Citrobacter rodentium*, *Klebsiella pneumoniae*, *Bacteroides* spp. and *Borrelia*) and Gram-positive (*Propionibacterium acnes*) bacteria, as well as fungi such as *Candida albicans*. Furthermore, IL-17 and Th17-cells are also discussed to be potent inducers of autoimmunity through the promotion of tissue inflammation and the mobilization of the innate immune system, since increased IL-17 levels have been observed in patients with rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease and psoriasis[105].

In contrast to Th17-cells, still little is known about Th9 effector cells. They are distinct from other T-cell subtypes through production of IL-9. Interestingly, TGF-β, the cytokine involved in Th17 differentiation, was shown to "reprogram" Th2 cell differentiation in presence of IL-4, leading to Th9 cells, loosing their Th2 characteristics, including expression of GATA-3. Similar to Th2 immune response, IL-9 is important in the immune defense against helminth infections and also appears during allergic reactions in the lung[110,111]. It is not clear if such plasticity between closely related effector programs is the norm, allowing "fine-tuning" of the immune response and if a reversal from Th9 to Th2 cells could also occur[111].

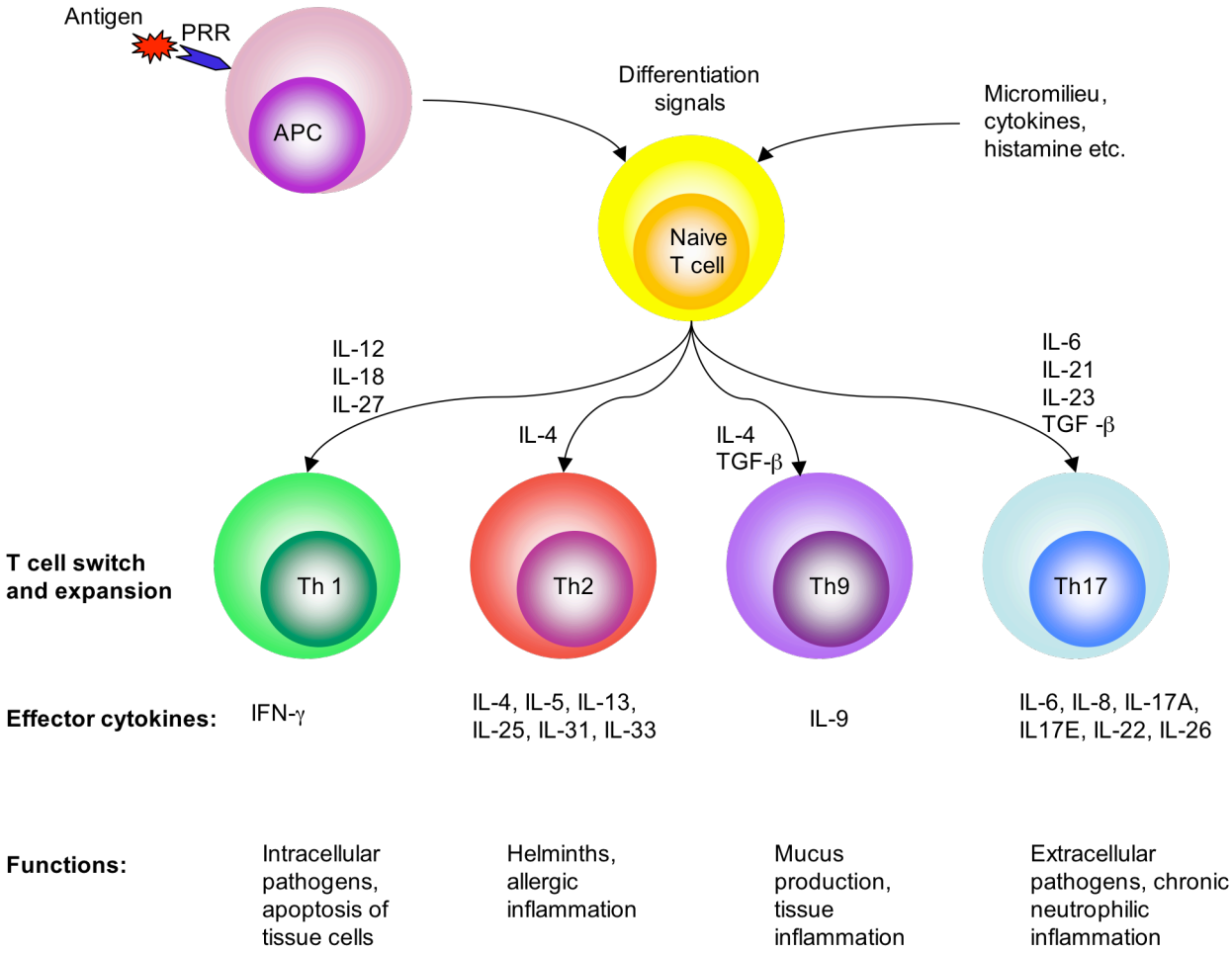


Figure 5. Effector T-cell subsets. After antigen presentation by DCs, naive T-cells differentiate into T_H1, T_H2, T_H9, and T_H17 effector subsets. Their differentiation requires cytokines and other cofactors that are released from DCs and also expressed in the micromilieu. T-cell activation in the presence of IL-4 enhances differentiation and clonal expansion of T_H2 cells, perpetuating the allergic response. IL-12, IL-18, and IL-27 induce T_H1-cell differentiation; IL-4 and TGF-β induce T_H9 differentiation; and IL-6, IL-21, IL-23, and TGF-β induce the differentiation of T_H17-cells. Adapted from Akdis C., and Akdis M. J Allergy Clin Immunol (2009), 123. 735-46

$CD4^+CD25^+$ Treg cells

5-10% of all $CD4^+$ T-cells in the blood and other peripheral lymphoid tissues and more than 20% in the bone marrow are $CD4^+CD25^+$ Treg cells. They express an α/β TCR repertoire with a high affinity for self-peptides[112]. For the development, maintenance and function of Treg cells the transcription factor forkhead box P3 (FOXP3) is required. Mice lacking FOXP3 (known as scurfy mice) as well as individuals without functional FOXP3 develop a severe autoimmune-like lymphoproliferative disease, indicating that Treg cells are primary mediators of peripheral tolerance[113]. Although FOXP3 is an invaluable marker of mouse Treg cells, it has been controversially discussed, if additional co- or transcriptional factors beside FOXP3 are also necessary in human Treg cell development and function, as a significant percentage of human activated T-cell express FOXP3 but do not possess regulatory activity[113,114]. Furthermore, in contrast to mouse Treg cells, induction of human Treg cells by stimulation with transforming growth factor (TGF) β does not confer a regulatory phenotype[113].

One suppression mechanism used by Treg cells is the secretion of inhibitory cytokines, such as IL-10 and TGF-β. IL-10 is known to be essential in peripheral tolerance to allergens, autoantigens, transplantation antigens, and tumor antigens and suppressive in T-cell proliferation and cytokine production[93]. Studies with allergy and asthma models have been shown that both naturally occurring and induced antigen-specific Treg cells control the disease depending on IL-10 and in some cases on IL-10 and TGF-β[93,113,115]. Additional suppression mechanisms by Treg cells are discussed[113], e.g suppression by Treg cell-mediated target-cell killing through secretion of granzyme A and perforin, following adhesion of CD18[116] or suppression of DCs through direct interaction between DCs and Treg cells, in process involving the co-stimulatory molecule cytotoxic T-lymphocyte antigen 4 (CTLA4), which is constitutively expressed by Treg cells[117,118]. However, the importance of different mechanisms in different cellular functions is not appreciable so far[113].

B-lymphocytes

Beside T-lymphocytes, B-lymphocytes are the second cell population involved in an adaptive immune response and are described briefly here.

B-lymphocytes maturate in the bone marrow, circulate as naïve B-cell in the blood and lymph and are carried to spleen and lymph nodes. They are characterized by their synthesis and display of antigen specific antibodies, encoded by heavy and light immunoglobulin (Ig) genes. The recognition of antigen by membrane bound antibodies leads to a rapid cell division and differentiation into effector cells, called plasma and memory B-cells. Sites of intensive B-cell proliferation are called germinal centers and are surrounded by Th-cells activating the B-cells and migrated with them. Plasma cells secrete soluble antibody

molecules with specificity for antigen that is identical to the surface receptor of the parent B-cell. Antibody molecules consist of two identical light polypeptide chains of about 22 kDa and two identical heavy chains of around 55 kDa or more. The antibody light chains are encoded by a variable (V), joining (J) and constant (C) gene segment whereas the heavy chain gene contains a V, D (diversity), J, and C gene segment. In mice and humans antibody diversity is generated by multiple germ-line gene segments, combinatorial V-(D)-J joining, junctional flexibility, P-region nucleotide addition, N-region nucleotide addition, somatic hypermutation and combinatorial association of light and heavy chains. Heavy chains consist of five different constant regions (μ, δ, γ, ε and α), which determine the antibody class (IgM (μ), IgG (γ), IgA (α), IgD (δ), or IgE (ε)) and effector function[4]. IgE are associated with allergic disorders and the features of allergic inflammation are described in more detail subsequently.

IgE driven immune responses and the development of allergies

To mount an IgE mediated immune response, first antigen presenting cells (e.g. dendritic cells) have to differentiate and to promote the formation of allergen-specific Th2-cells, a process that may be enhanced by engagement of Notch at the surface of T-cells with the Notch ligand Jagged on dendritic cells. Th2-cells subsequently stimulate B-cell to IgE class switch by secretion of IL-4 and IL-13 and by ligation of suitable co-stimulatory molecules

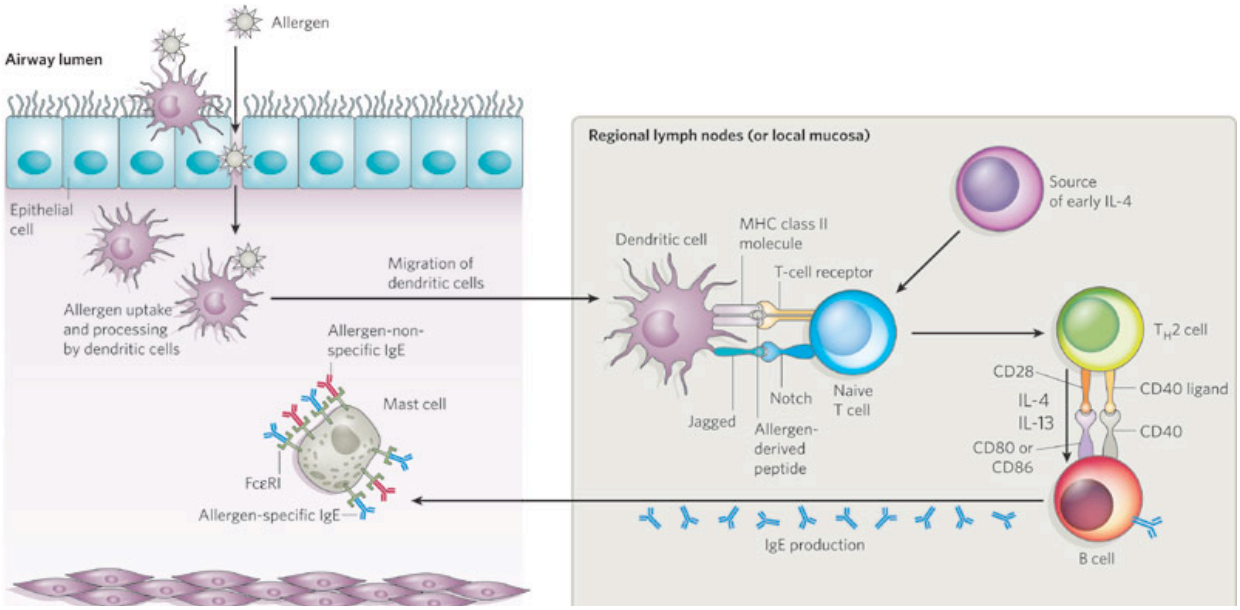


Figure 6. Sensitization to allergens in the airway. DCs become activated after allergen uptake and migrate to regional lymph nodes or to sites in the local mucosa, where they present allergen peptides on MHCII molecules to naïve T-cells. In the presence of IL-4, naïve T-cells acquire the characteristics of TH2-cells, a process that may be enhanced by engagement of Notch at the surface of T-cells with Jagged on DCs. TH2-cells produce IL-4 and IL-13. In the presence of these cytokines and the ligation of suitable co-stimulatory molecules (CD40 with CD40 ligand, and CD80 or CD86 with CD28), B-cells undergo Ig-class-switch recombination, and IgE antibodies are produced. Basophils and mast cells also can produce IL-4 and/or IL-13, and can stimulate B-cells through CD40 (not shown). IgE diffuses locally and enters the lymphatic vessels. It subsequently enters the blood and is then distributed systemically. After gaining access to the interstitial fluid, allergen-specific or non-specific IgE binds to the high-affinity receptor for IgE (FcRI) on tissue-resident mast cells, thereby sensitizing them to respond when the host is later re-exposed to the allergen. Adapted from Galli SJ, et al. Nature 2008; 454(7203):445-54.

(CD40 with CD40 ligand, and CD80 or CD86 with CD28) and recruit eosinophils through releasing of IL-5, IL-9, and granulocyte/macrophage-colony stimulating factor (GM-CSF). B-cells are also stimulated by IL-4 and/or IL-13 producing basophils and mast cells through CD40[119]. Allergen-specific or unspecific IgE diffuses locally, enters the lymphatic system and the blood and is distributed systemically. It binds to the high-affinity receptor for IgE (FcεRI) on mast cells, thereby sensitizing them (Fig. 6). Sensitization does not produce symptoms (for example, if sensitization occurs by way of the airways, bronchoconstriction does not occur). However, a later re-exposure to the same allergen induces cross-linking of receptor-bound IgE with subsequent mast cell degranulation and the release of proinflammatory molecules such as histamines. Such an acute reaction is known as early-phase reaction or a type I immediate hypersensitivity reaction and causes vasodilation, increased vascular permeability with oedema, and acute functional changes in affected organs. During the course of the reaction often mediators are released to promote the local recruitment and activation of leukocytes, leading to late-phase reactions[4,119,120]. Late-phase reactions typically occur 2-6 h after allergen exposure and often peak after 6-9 h. The reaction is characterized by infiltration of cells of the innate and adaptive immune system (neutrophils, eosinophils, macrophages, Th2-cells, and basophils) and persistent mediator production by resident cells, such as mast cells[4,119].

Allergic diseases and the "Hygiene Hypothesis"

Over the past two to three decades the prevalences of allergic diseases (also known as atopic diseases) including allergic rhinitis (also referred as hay fever), atopic dermatitis (also known as eczema), allergic (or atopic) asthma and some food allergies has been significantly increased in the western world[119-122]. Currently, more than 130 million people suffer from asthma. Furthermore, in the wake of improved hygiene and socio-economic conditions as well as antibiotics and vaccinations, the incidence of many infectious diseases has decreased. In contrast, the prevalence of allergic diseases is considerably lower in developing countries[121,122].

The hygiene hypothesis states that a lack of infections and unhygienic contact early in childhood increases the risk of developing allergic diseases and was first proposed by Strachan in 1989. He observed that the prevalences of hay fever and eczema was inversely related to the number of siblings in a family suggesting that unhygienic contact to older siblings transmit infection in early childhood, which leads to protection against allergic disease[123]. It has been suggested that the limited exposure to bacterial and viral organisms during early childhood results in a shift to Th2-cell expansion through insufficient stimulation of Th1-cells. In fact many epidemiological studies have shown that children growing up on a farming environment rich in bacterial endotoxin (e.g. the TLR4 ligand LPS) or living an

anthroposophic lifestyle are more protected in developing hay fever and atopic sensitization[124-127]. Environmental factors correlate with the gene expression of CD14, TLR2 and TLR4[128,129], and were related to different farm animal species the mother has contact with during pregnancy in a dose-dependent manner[128]. Additionally, children with contact to farm animals, consumption of farm milk or haying, have a significantly higher expression of different TLRs and CD14[130]. The manifestation of childhood asthma and allergies occurs in the first years of life [131]. Therefore pre- and early postnatal exposure seems to determine the development of childhood asthma and allergies[131]. In chapter A1 we investigated the influence of farming exposures during pregnancy and in the first year of life on the expression of different TLRs and CD14.

Allergic diseases and genetic associations

The innate immune system is an important mediator between microbial exposure and regulation of cytokine response and subsequent development of asthma and allergy. Different TLR polymorphisms are identified, which seem to be involved in pathogenesis of asthma. Results of the ALEX (ALlergy and EndotoXin) study have shown that farmer′s children carrying one or two *TLR2* – 16934T alleles had significantly reduced prevalences of asthma, fewer current asthma symptoms, less hayfever and atopy[132]. The *TLR2*/+596C polymorphism was found to be associated with an increased risk for asthma[133]. For a *TLR4* genetic variant (*TLR4*/+4434) a gene-environment interaction was found, since only farmer′s children heavily exposed to endotoxin showed reduced atopy, whereas this effect was reversed in low endotoxin exposure[132]. Polymorphisms of *TLR1* (*TLR1*/-2192 C/T; *TLR1*/743 A/G) *TLR6* (*TLR6*/-2078 T/A) and *TLR10* (*TLR10*/2323 A/G) were shown to be highly significant, inversely associated with atopic asthma in a case-control study[134]. The CD14/-260T allele is described to protect against asthma and asthma severity in family-based and case-control studies[135]. The well-studied CD14/-260C allele has been shown to be negatively associated with asthma in skin prick test positive subjects[133] and children with regular contact with pets have higher levels of both total and specific serum IgE to aeroallergens, whereas children with regular contact to stable animals have an opposite association[136], indicating that the type and concentration of microbial molecules to which children are exposed may influence the genetic associations observed. Behind them, differences in timing of exposure might be one explanation for different results between studies[135]. We investigated in chapter A2 which factors, polymorphisms or a genotype-prenatal farm environment interaction, could influence the TLRs gene expression and investigated whether the TLRs and CD14 gene expression in cord blood samples is associated with the development of atopic dermatitis in the first 2 years of life.

Studying the protective factors against childhood allergies: The ALEX-, Parsifal-, and PASTURE Study

In the past, different epidemiological studies aimed to identify not only risk but also possible protective factors against allergic diseases. Results of two European cross-sectional studies were meaningful: The ALEX (Allergy and EndotoXin) study, which investigated the environmental exposure to endotoxin during pregnancy and first year of life and its relation to allergic diseases[137] as well as the PARSIFAL (Prevention of Allergy Risk factors for Sensitization in children related to Farming and Anthroposophic Lifestyle) study which compared children from farm or anthroprosophic families with appropriate reference children from Austria, Germany, Netherlands, Sweden and Switzerland[138]. However, both studies assessed the early exposure retrospectively. To investigate the temporal sequence of events, relating early life exposures to the maturation of the immune response and the development of tolerance and allergies, respectively, the PASTURE (Protection against Allergy – Study in Rural Environment) study was developed. This prospective birth cohort study located in rural areas in five European countries (Germany, Austria, Switzerland, France and Finland) has the overall aim to assess the role of indoor exposure to various microbial products for the development of childhood asthma and allergies and to investigate the immunological and genetical mechanisms which are involved in the determination of individual responses to these environmental influences[139]. Our investigations, described in chapters A1-A3, are based on the PASTURE study.

Food allergy: cause and treatment

Immediately after birth, the mucous membrane of the gastrointestinal tract is colonized by a large variety of microorganisms and environmental antigens. There are numerous mechanisms to protect the body from invasion by potentially pathogenic microorganisms: Behind the physical barrier, the production of secretory IgA seems to be particularly important. Specialized epithelial cells, so called M cells, but also enterocytes, or dendritic cells take up dietary proteins in the gut and presented them as fragment to naïve T-cells in the gut-associated lymphoid tissue (GALT)[4]. Healthy individuals exhibit a transient proliferative response to food antigens, but non-responsive (oral tolerance), whereas atopic individuals develop an exaggerated Th2 response to specific dietary antigens[140]. Up to 6% of children under 4 years of age and 3-4% of adults in westernized countries suffer from clinical symptoms of food allergy and their prevalence appears to be rising[141]. Many of them have IgE-mediated food allergy that may lead to a fatal outcome or to life-threatening conditions such as anaphylactic shocks (49% of life-threatening cases), systemic reactions (33%), laryngeal angiodedema (13%) or acute asthma (5%)[142]. The cause of food allergy is still unclear. There is evidence that food allergy is at least in part genetically determined. Peanut

allergy, for example, is about tenfold more likely to occur in a child with a parent or sibling who is peanut allergic compared to the general population risk[143]; An *IL10 gene* polymorphism has been found to be associated with food allergy in a Japanese population[144] and an *IL13 gene* polymorphism has been associated in German children[145]. There are many epidemiological studies examining the prevention of allergic diseases, however little work has been done with respect to the hygiene hypothesis and food allergy. Recently, experimental findings of *in vivo* studies have shown that neonatal mucosa exposure to *Staphyloccocus aureus* reduces the risk of developing food allergy[146].

The current treatment of food allergic diseases is to avoid trigger foods and to treat allergic reactions. Different therapies are being evaluated to treat or prevent food allergies: Attempts of subcutaneously delivered native peanut allergen resulted in modest clinical improvements and significant adverse anaphylactic effects[147]. Further therapies are in clinical or preclinical studies including peptide vaccination, plasmid-DNA encoded vaccines, modified protein vaccine, anti-IgE or cytokine/anticytokine treatment[143]. We observed some promising results in murine *in vivo* studies with the use of attenuated, intracellularly living bacteria which deliver allergen in small concentration (chapter B1 and B2).

Aims and objective

Results of retrospective analysis provided first evidence that stimulation with farming factors during pregnancy and early in life affects the innate immunity and development of allergic diseases. In **Chapter A1** we investigated the influence of farming exposures during pregnancy and in the first year of age on the expression of TLRs and CD14 as important receptors of the innate immunity, at time of birth and at the age of one. The analysis was carried out with samples of farming and non farming families living in rural environments across five European countries within the prospective longitudinal birth cohort study PASTURE

The hygiene hypothesis has proposed that insufficient stimulation of the immune system may influence the development of allergies. In **Chapter A2** we analyzed the association of TLRs and CD14 gene expression at time of birth and the development of atopic dermatitis in the two first years of life. Furthermore, we investigated, if the TLRs/CD14 gene expression could be influenced by genetic factors (single nucleotide polymorphisms) or by a gene-environment interaction.

Recent studies indicate that prenatal, but not postnatal, vitamin D intake protects from the development of wheeze and atopic diseases in 3 and 5 years old children. Vitamin D, has a immunmodulatory capability and is able to induce tolerogenic dendritic cells (DCs). These cells are characterized to upregulate two inhibitory receptors immunglobulin-like transcripts

(ILT)3 and ILT4, which inhibit nuclear factor (NF)-κB activation and the subsequent inflammatory responses. In addition, tolerogenic DCs are able to induce regulatory T cells (Treg), render naïve T cells anergic, and are therefore interesting candidates in the development of atopic diseases. To evaluate, if prenatal vitamin D supplementation could induce tolerogenic DCs at birth, we analyzed in **Chapter A3** the gene expression levels of ILT3 and ILT4 in cord blood samples of the PASTURE population.

The increased incidence of food allergy in developed countries is in particular serious, because of missing therapeutic and preventive strategies beside the avoidance of triggering foods. We aimed to develop an allergen-specific immune therapy, where patients are treated with increasing concentrations of the allergen and which is already established against hay fever or insect sting allergies. As a tool to release the allergen in small concentration we used intracellular living attenuated bacteria (*Salmonella enterica* serovar Typhimurium). First, we studied the allergen expression in different attenuated *Salmonella* strains and investigated in a preliminary *in vivo* mouse model the influence of pre-treatment with such bacteria on the development of allergic symptoms (**Chapter B1**). Next, we tested, in two murine sensitization studies intensively the effect of combined intake of microorganisms and the release of low allergen amounts in a preventive as well as in a therapeutical approach (**Chapter B2**).

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CHAPTER A1

Prenatal and early life factors associated with expression of innate immunity genes at birth and at year 1

Written as manuscript for publication

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Abstract

Background

Decreased microbial exposure may be one reason for increased incidence of allergic disorders in westernized countries.

Objective

We investigated the influence of prenatal and early postnatal farming exposures on the expression of innate immune receptors (Toll-like receptors and CD14) at time of birth and at one year of age.

Methods

The prospective birth cohort study (PASTURE) comprises 530 children of farming and 603 children of non-farming families, and their mothers from rural areas of 5 European countries (Austria, Finland, France, Germany and Switzerland). Gene expression of Toll-like receptors (TLRs) and CD14 were analyzed at time of birth and at one year of age. Mothers answered questionnaires about health issues, farming exposure and potential confounders such as active and passive smoking, parental education and family size within the third trimester of pregnancy as well as two and twelve month after birth.

Results

The expression of TLR7 and 8 were significantly higher in farmer children compared to non farmer children at time of birth. The same trend was observed for TLR 1, 2, 4, 5, 6, and 9.
At one year of age TLR5 and 6 were significantly higher expressed in children, who consumed farm milk during the first year of age. Consumption of unboiled farm milk was positively associated with CD14 and TLR4, 5, 6, and 7.
Consumption of unboiled farm milk compared to consumption of no farm milk showed a positive association with all tested differences of TLR expression (diff-TLR) between time of birth and one year of age and a significant result was observed for diff-TLR6. If the first unboiled farm milk intake occurs in the first 10 months a significant effect was observed for diff-TLR6 and 7 and a nearly significant effect for diff-TLR1 and 4 but not if the first intake occurs between 10 and 12 month of life.

Conclusion

Working on a farm during pregnancy influences the expression of different TLRs and CD14 in cord blood. Farm milk, especially unboiled farm milk consumption during the first 12 months of life is associated with TLRs expression at one year of age. It seems that both the pre- and early postnatal farming exposures influence the expression of TLRs in children.

Introduction

The prevalence of allergic diseases has increased and stabilized on a high level in wealthy countries in recent decades[1-6]. These observations may be based on decreased exposures to endotoxin caused by modern public health practices, suggesting that microbes or microbial components are important stimuli for immune education and protection against allergic diseases.

Immune responses to microorganisms are mediated by receptors of the innate immunity such as Toll-like receptors (TLRs) and CD14. TLRs are highly conserved, germ-line encoded receptors located on the cell surface or intracellularly on endosomal membranes of different immune, but also epithelial and endothelial cells and recognize conserved microbial structures[7]. The farming environment was shown to be rich of different bacterial components like lipopolysaccharid (LPS) or N-acetyl-muramic acid[8,9] both ligands, which are recognized by TLRs and CD14. A direct influence of farming exposition on the expression of TLR2 and CD14 was found to be increased in children living on a farm compared to non-farmer children[10]. Children with contact to farm animals, consumption of farm milk or haying, have a significantly higher expression of different TLRs and CD14[11]. Furthermore, maternal exposure to different farm animals during pregnancy increased the expression of TLR2, TLR4 and CD14[12], indicating that the development of the infantile innate immune system is affected through the maternal farming exposition. However, these results are based on retrospective epidemiological studies with school-aged children. To maintain further insights in the importance of early prenatal and postnatal farming stimulation and the consequence on the development of allergic diseases, the prospective longitudinal birth cohort study PASTURE (Protection against allergy: study in rural environment) was established which includes farming and non farming families living in rural environments.

The aim of the present study was to investigate the influence of farming exposures during pregnancy and in the first year of age on the expression of TLRs and CD14 at time of birth and at the age of one.

Methods

Population and study area

PASTURE is a prospective birth cohort study located in rural areas in five European countries: Austria, Finland, France, Germany and Switzerland. This present analysis utilizes data of altogether 1133 children, 530 children of farming and 603 children of non farming families, and their mothers.

Potential participating families were contacted in the third trimester of pregnancy[13]. Exclusion criteria were living on a farm without livestock, maternal age below 18 years, premature

delivery, genetic disease in the offspring, absent telephone connection and insufficient knowledge of the country's language. Women living on a farm where livestock was held or who or whose partners actively run a farm were considered farming women. As controls a subset of the population living in close neighborhood in likewise rural environment not occupationally involved in farming activities was assigned.

Questionnaires

Within the PASTURE study group an extensive questionnaire was developed using questions from the International Study of Allergy and Asthma in Childhood (ISAAC)[14], the Allergy and Endotoxine (ALEX) study[15] the PARSIFAL (Prevention of Allergy-Risk factors for Sensitization in children related to farming and anthroposophic lifestyle) study[16], as well as questions derived from the American Thoracic Society (ATS) questionnaire[17] for the assessment of respiratory health in the parents. The questionnaires were administered by interview to the mother of the child within the third trimester of pregnancy and two and twelve month after birth of the study child. Questions were designed to assess respiratory and other health issues of the mother as well as a range of agricultural exposure. In addition, questions about potential confounders such as active and passive smoking, parental education and family size were included in the questionnaires.

Expression of Toll-like receptors and CD14

Cord blood was taken by the midwives with PaxGene tubes shortly after cord clamping. Blood samples at year one were taken during a field visit from the basilic or hand vein. The blood was frozen at minus 80°C within twenty-seven hours after delivery at the local laboratory. At a central laboratory total mRNA was isolated with the PaxGene Blood RNA Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen) and stored at minus 80°C. For reverse transcription (RT), 600ng of total RNA in a final volume of 60µl were used and adequate amounts of TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, USA) were added. The running conditions were as follows: 10 min 25°C; 40 min 42°C; 5 min 95°C. The gene quantification was performed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) using the TaqMan® low density array (LDA) system of Applied Biosystems with the following running conditions: 45x (2 min 50°C; 10 min 95°C; 15 sec 95°C; 1 min 60°C). The used predeveloped primer/probes (Applied Biosystems) are listed in Table 1. The determined gene expression values were normalized to the parallel measured endogenous controls 18S rRNA and beta-2-microglobulin. The data was analyzed using the comparative Ct method according to the manufacturer's instructions (Applied Biosystem).

Table 1: Primer/probe assay

Assay ID	Gene
Hs00413978_m1	TLR1
Hs00610101_m1	TLR2
Hs00152933_m1	TLR3
Hs00370853_m1	TLR4
Hs00152825_m1	TLR5
Hs00271977_s1	TLR6
Hs00152971_m1	TLR7
Hs00607866_mH	TLR8
Hs00370913_s1	TLR9
Hs00169122_g1	CD14
Hs99999907_m1	β2 microglobulin
Hs99999901_s1	18S rRNA

Statistical analyses

Frequencies of the characteristics of the pregnant women and the children were compared according to farming classification by Chi^2-test.

To quantify the results obtained by real-time RT-PCR for CD14 and TLR1, 2, 4, 5, 6, 7, 8. 9, the comparative threshold method, described by Giulietti and colleagues, was used[18]. We choose a non farmer child with results above detection limit for all mRNA-measurements in cord blood and at year one as a reference. The results give a multiple of amount of mRNA in comparison to the reference. Because the distribution of the transformed gene expression levels was skewed, the calculated variables were log-transformed (natural logarithm), resulting in a good approximation to normal distribution.

The transformed data were used in linear regression to calculate associations between mRNA expressions and farming or other exposures. To assess the development of mRNA expressions from birth to year one, the difference of normalized mRNA expressions from cord blood and year one was used (diff-mRNA). Mean ratio (mr) and p-values are given. P-values <0.05 were considered significant.

Associations between mRNA expressions and exposures were calculated in bivariate regression models.

An overall association of farming status on mRNA expressions in cord blood and farm milk consumption on the set of mRNA expressions at year 1 or diff-mRNA respectively was tested using multivariate analyses of variance (manova). Given the values for Wilks' lambda and the level of significance outcome models for singular mRNA expressions were accordingly classified and interpreted.

All of the statistical analysis was performed using STATA 10.

Quality control

Extensive quality control measures have been incorporated in the PASTURE cohort study particularly for laboratory work but also for field work.

Ethical approval

The study was approved by the ethical boards of the five study centers, and written informed consent was obtained from the children's parents for questionnaires, blood samples and genetic analyses.

Results

Of 2871 women with response after contacted for the PASTURE study 1772 (62%) were identified as eligible (Fig 1). 64% of these women were willing to participate and included in the study (530 farming and 603 non farming women). For mRNA analyses 938 (52.5% non farming, 47.5% farming children) cord blood samples (82.3% of study population) and 773 (51% non farming, 49% farming children) blood samples of year one (68.3% of study population) were available. Table 2 shows environmental and farming characteristics of pregnant women and children in first year of life.

The study-population and the populations with available mRNA measurements at birth and year one did not differ in respect to farming status (data not shown). Compared to the study population a higher percentage of Finnish and French blood samples were available. No differences were seen with respect to age of pregnant mothers, her educational level, number of older siblings, smoking status of the mother, pet owner ship, family history of asthma, hay fever and eczema or the prevalence of farm milk consumption.

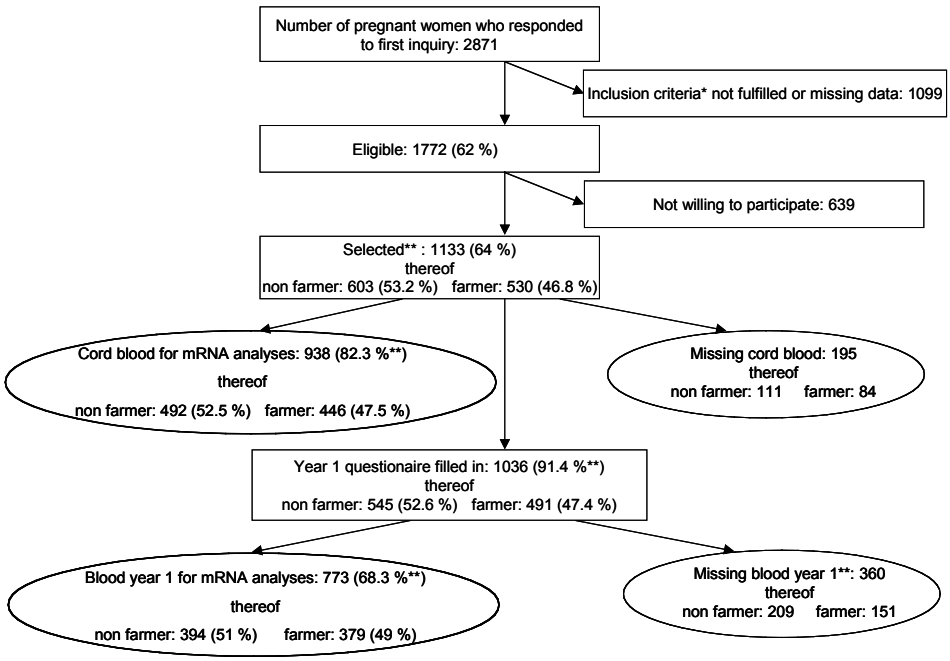

Figure 1. Selection of PASTURE study population and participants for mRNA analyses

* Inclusion criteria were: living on a farm with livestock, maternal age above 18 years, term delivery, no genetic disease in the offspring, telephone connection, and sufficient knowledge of the country's language.

**selected: pregnancy questionnaire filled in.

Heterogeneity of TLR expression between centers

Comparison of centers revealed heterogeneity of mRNA expression between centers depending on farming status. Particularly Switzerland (CD14, TLR7 and 9) and Finland (TLR1, 2, 4, 5, 6 and 8) had an inverse effect of farming status on mRNA expressions in cord blood. Deeper exploration of differences between centers did not show any farming activity responsible for the contrary effects but an interaction for farming status between the Finish and Non-Finnish population.

	Farmer		**Non Farmer**		**Chi2**
spalte1	N	%	N	%	p-value
population at birth	530	46.8	603	53.2	
population at year1 (percent of population at birth)	491	92.6	545	90.4	
male	266	50.2	293	48.6	0.966
center					
Austria	105	47.7	115	52.3	
Switzerland	107	44.2	135	55.8	
France	94	46.3	109	53.7	0.389
Germany	112	44.1	142	55.9	
Finland	112	52.3	102	47.7	
specific farming exposure of child					
in utero through maternal exposure					
regular stay in stable: yes	462	87.2	107	17.7	<0.001
regular stay in barn: yes	360	67.9	65	10.8	<0.001
maternal farm milk consumption: yes	406	76.6	98	16.3	<0.001
only boiled fmilk	94	17.7	27	4.5	<0.001
any unboiled fmilk	310	58.5	70	11.6	<0.001
contact to >= 2 animal species	196	37.0	61	10.1	<0.001
during year1*					
child lived/or parents worked on farm: yes	484	98.6	10	1.8	<0.001
regular contact to farm: yes	485	98.8	78	14.3	<0.001
regular contact to stable: yes	331	67.4	41	7.5	<0.001
farm milk consumption: yes	280	57.0	56	10.3	<0.001
only boiled farm milk	139	28.3	27	5.0	<0.001
any unboiled farm milk	139	28.3	24	4.4	<0.001
unboiled farm milk before month 1-9	58	11.8	8	1.5	<0.001
first unboiled farm milk month 10-12	78	15.9	15	2.8	<0.001
general exposures of child					
maternal history of asthma: yes	38	7.2	61	10.1	0.08
maternal history of hay fever: yes	108	20.4	196	32.5	<0.001
paternal history of asthma: yes	28	5.3	43	7.1	0.15
paternal history of hay fever: yes	83	15.7	188	31.2	<0.001
>= 2 older siblings	255	48.1	124	20.6	<0.001
maternal smoking during pregnancy: yes	46	8.7	112	18.6	<0.001
maternal smoking while breast feeding*: yes	17 38	3.2	38	6.3	0.01
cat and/or dog in pregnancy: yes	430	81.1	233	38.6	<0.001
cat and/or dog during year1*: yes	402	75.8	191	31.7	<0.001
at least 4 month breast feeding*: yes	301	56.8	334	55.4	0.962

Table 2. Environmental and farming characteristics of pregnant women and children in first year of life, by farming status.

*percent of population at year 1.

Associations of farming status and TLR/CD14 expressions at birth

The expression of TLR7 and 8 were significantly higher in cord blood of farmer compared to non farmer children (Fig. 2). The same but not significant trend was observed for TLR 1, 2, 4, 5, 6, and 9.

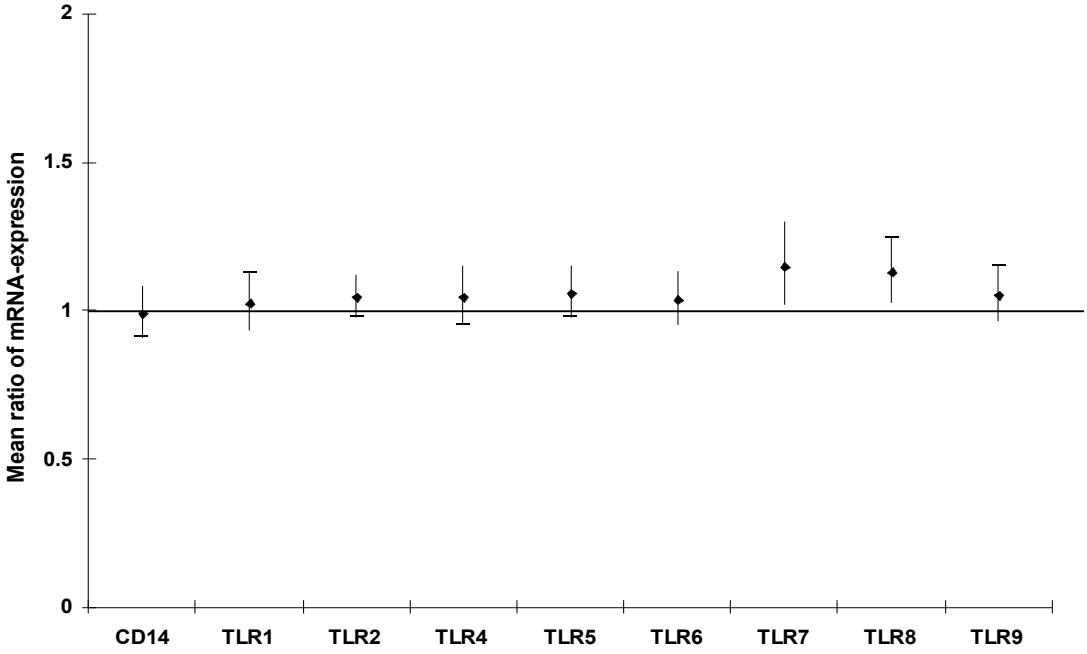

Figure 2. Association of farming/non farming status and mRNA-expressions (normalized to 18s/b2m) at birth, adjusted for maternal history of asthma and hay fever, sex, maternal smoking in pregnancy, and center. Shown are mean ratios, whiskers represent the 95% confidence intervals. Wilks' lambda $F_{(1,1)}$= 1.90 and p-value=0.048.

Association of maternal exposure on Toll-like receptors and CD14 at birth

Maternal smoking during pregnancy led to decreased expression of TLRs, especially TLR6 and 8, which were reduced significantly. Other exposures during pregnancy (regular stay in stable, farm milk consumption, contact to less or more than two animal species, older siblings) did not reveal significant associations for more than one mRNA expression at birth (data not shown).

Association of farm milk consumption at year 1 and expression of Toll-like receptors and CD14 at one year of age

Children who consumed farm milk during the first year of age have significantly higher expression of TLR5 and 6. Consumption of unboiled farm milk was positively associated with CD14 and TLR4, 5, 6, and 7 (Table 3). Other positive associations were observed when

older siblings were existent (significant for CD14, TLR4, 6) or mother smoked during lactation (CD14, TLR4). Farming status or regular contact to farm was not associated with mRNA expressions at year one (data not shown).

	cd14_1		tlr1_1		tlr2_1		tlr4_1		tlr5_1		tlr6_1		tlr7_1		tlr8_1		tlr9_1	
	mr	p-value	mr	p-value	mr	p-value	mr	p-value	mr	p-value	mr	p-value	mr	p-value	mr	p-value	mr	p-value
farm milk consumption: yes/no	1.07	0.148	1.04	0.472	1.04	0.421	1.08	0.181	1.11	0.050	1.10	0.042	1.10	0.132	1.04	0.546	1.10	0.128
only boiled farm milk vs no farm milk	0.98	0.798	0.95	0.451	1.0	0.990	0.92	0.286	1.04	0.620	0.99	0.831	0.97	0.745	0.93	0.371	1.05	0.544
any unboiled farm milk vs no farm milk	1.15	0.025	1.13	0.80	1.06	0.299	1.26	0.002	1.18	0.017	1.23	0.001	1.25	0.007	1.15	0.074	1.17	0.066

Table 3. Associations* of exposure during year 1 and mRNA-expression** at year 1

*Linear Regression: bivariate model; ** normalized for 18s/b2m,; mr=mean ratio.

Wilks' lambda $F_{(1,1)}$=2.1, p-value=0.073 (results of multivariate analysis of variance (manova) of mRNA expression and farm milk consumption (unboiled farm milk/no or only boiled farm milk) adjusted for center).

Farm milk consumption during first year of life and differences of TLR expression between cord blood and one year of age (diff-mRNA)

Consumption of unboiled farm milk compared to consumption of no farm milk showed a positive association with all tested diff-mRNA with a significant result for diff-TLR6. When the first intake of unboiled farm milk was taken in the first 10 month a significant effect was observed for diff-TLR6 and 7 and a nearly significant effect for diff-TLR1 and 4. These results disappeared if the first intake occurs between 10 and 12 month of life (Table 4).

	diff cd1 4		diff tlr1		diff tlr2		diff tlr4		diff tlr5		diff tlr6		diff tlr7		diff tlr8		diff tlr9	
	m r	p-value	m r	p-value	m r	p-value	m r	p-value	m r	p-value	m r	p-value	m r	p-value	m r	p-value	m r	p-value
farm milk consumption: yes/no	1.13	0.32 0	1.08	0.52 5	1.03	0.79 3	0.99	0.96 9	0.92	0.48 3	1.17	0.20 3	0.99	0.95 4	0.97	0.82 7	1.04	0.81 9
only boiled farm milk vs no farm milk	1.10	0.55 4	0.90	0.48 3	0.97	0.84 7	0.78	0.14 3	0.83	0.24 0	0.92	0.58 6	0.78	0.23 6	0.89	0.42 4	0.98	0.92 2
any unboiled farm milk vs no farm milk	1.14	0.39 5	1.21	0.19 6	1.05	0.71 2	1.19	0.29 0	0.95	0.74 1	1.41	0.02 3	1.19	0.40 1	1.00	0.98 2	1.05	0.78 4
unboiled farm milk vs no/only boiled farm milk	1.11	0.49 4	1.26	0.09 8	1.06	0.63 8	1.31	0.09 0	1.18	1.01	1.46	0.00 8	1.30	0.17 5	1.05	0.74 0	1.06	0.74 0
first unboiled farm milk before month 10	1.05	0.82 5	1.39	0.09 8	1.26	0.20 1	1.44	0.10 4	1.21	0.37 2	1.64	0.01 6	1.92	0.02 0	1.23	0.30 5	1.11	0.68 8
first unboiled farm milk month 10-1 2	1.14	0.46 0	1.21	0.26 3	0.95	0.75 6	1.24	0.26 0	0.91	0.60 9	1.38	0.06 6	1.03	0.89 7	0.96	0.79 2	1.03	0.87 8

Table 4. Associations* of farm milk consumption in the first year of life and difference of mRNA expression** (diff-RNA) between cord blood and year 1.

* Linear regression adjusted for being a farm child, maternal history of asthma, maternal history of hay fever, sex, number of older siblings, and center; **normalized for 18s/b2m; mr=mean ratio.

Wilks' lambda F(1,1)= 2.8, p-value=0.003 (results of multivariate analysis of variance (manova) of difference of mRNA expression and farm milk consumption (unboiled farm milk/no or only boiled farm milk) adjusted for maternal history of asthma, maternal history of hay fever, sex, and center)

Discussion

In contrast to previous retrospective epidemiological analysis, we were able to demonstrate in a prospective approach that the expression of different Toll-like receptors (TLRs) and CD14 in cord blood of children whose mothers worked on a farm during pregnancy were partly significantly higher compared to non farming newborns. Furthermore, the TLRs expression at one year of age was associated with farm milk consumption, especially unboiled farm milk. This indicates that both the pre- and early postnatal farming exposure influence the expression of TLRs in children. In fact, numerous epidemiological studies have shown that growing up on a farm protects against the development of hay fever and atopic sensitization in childhood and adulthood[15,19-24]. Consumption of unpasteurized farm milk[12,15,25], exposition to animal stables[15,23] and contact to different farm animal species[12] mainly during pregnancy or early in life have been identified as important beneficial factors against allergic diseases.

TLRs as receptors of innate immunity are an important link between environmental exposure and initiation of subsequent adaptive immune responses[26,27]. Stimulation of TLR2 and TLR4 in a murine allergy model resulted in a decrease of allergic inflammatory response, mainly if the stimulation occurs before sensitization[28]. Further data of the Pasture study have shown

that children with atopic dermatitis in the first two years of life have a significant decreased expression of TLR5 and 9 in cord blood. These differences are not based on genetic modifications but on gene-environment interaction since the farming status increases significantly the expression of both genes among children with a genetic predisposition (unpublished data, chapter A2). These results indicate that the innate immunity is at least in part an important mediator for protection against allergic diseases.

However, two important points have to be resolved: First, since we did not find a single exposition factor, it still remains to clarify which combinations of exposures are relevant to influence TLR expression effectively and second, how TLRs stimulated by farming factors can modulate the subsequent immune cascade. The earlier proposed concept that insufficient immune stimulation disturbs Th1/Th2 balance in allergic diseases, appears deficient to explain many experimental and clinical observations, where both T-cell subtypes are involved[29-31]. In the last decade the scientific interest on T regulatory cells (Tregs) and their function to balance immune responses and maintaining peripheral tolerance has risen. Children who outgrew their cow`s milk allergy were found to have higher circulating $CD4^+$ $CD25^+$ Treg cells compared to children who maintained clinically active allergy[32]. It was shown that the expression of the important Treg cell marker transcription factor forkhead box protein P3 (FOXP3) negatively correlates with IgE, eosinophilia, and IFN-γ levels, and patients with asthma and atopic dermatitis have had a significant lower $FOXP3^+/CD4^+$ ratio compared to healthy individuals[33]. Furthermore, it was found that the influence of prenatal farm exposures results in an increased number of Treg cells in cord blood and reduced Th-2 cytokine production and the number of animal species increased FOXP3 expression[34].

How prenatal farm exposure and the exposition to farm milk early in life influence the expression of innate immune receptors and further modulate adaptive immune responses, especially the promotion of Treg cells, requires further work.

Contribution to this work

The PASTURE project was planned and is coordinated by E. von Mutius et al.[13]. RNA-isolation, reverse transcription and gene expression measurements were done by myself at the Children's Hospital Zürich. Statistical analyses were performed by Sondhja Bitter and Letizia Grize at the Institute of Social and Preventive Medicine (University of Basel). The manuscript was written by myself.

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CHAPTER A2

Children with Atopic Dermatitis in early life have reduced gene expression of innate immunity receptors at birth

Written as manuscript for publication

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Abstract

Background

Based on the hygiene hypothesis, it has been suggested that an insufficient stimulation of the immune system might contribute to the development of allergic diseases. Epidemiological evidence supporting the association with atopic dermatitis remains inconsistent.

Objective

We studied whether the gene expression of innate immunity receptors (Toll-like receptors, TLRs) in cord blood samples is associated with the development of atopic dermatitis in the first 2 years of life. We further investigated which factors, polymorphisms or a genotype-prenatal farm environment interaction, could influence the TLRs gene expression.

Methods

961 children participated in the prospective longitudinal birth cohort study PASTURE (Protection against allergy: study in rural environment) from rural areas of 5 European countries (Austria, Finland, France, Germany and Switzerland). Doctor diagnosis and symptoms of atopic dermatitis were reported by the parents from 1 to 2 years of age by questionnaires. Data on farm exposures during pregnancy were obtained from the questionnaire completed at the end of the pregnancy. Gene expression of TLRs and CD14 as well as polymorphisms in TLRs were assessed in cord blood of these children.

Results

The expression of TLR5 and TLR9 was significantly reduced in children with atopic dermatitis in the first 2 years of life compared to children with no dermatitis. The same tendency was observed with TLR1, 2, 4, 6, 7, 8 and CD14. The children with an expression of TLR5 or TLR9 in the upper tertile had a 50% reduced risk to develop atopic dermatitis in the first 2 years of life (adjusted OR and 95%CI: TLR5: 0.51, 0.32 to 0.81; TLR9: 0.47, 0.30 to 0.75) compared to children with an expression in the lower tertile. Prenatal farming environment such as maternal contact to farm animal during pregnancy had a protective effect on atopic dermatitis in the first 2 years of life. Polymorphisms in TLR5 and TLR9 did not modify the level of TLR5 and TLR9 mRNA from cord blood samples. However, among farmer children, TLR5 and TLR9 mRNA expression level was higher compared to non-farmer children with the respect of TLR polymorphisms.

Conclusion

Children with higher expression of TLR5 and TLR9 in cord blood have a reduced risk of atopic dermatitis in the first 2 years of life. The upregulation of these receptors of the innate

immunity might be mediated by the interaction between genotypes and prenatal farming environment.

Introduction

Atopic dermatitis is a chronic inflammatory, pruritic, skin disease, often occurring in early infancy, that affects up to 20% of the children in industrialized countries[1]. Asthma develops in approximately 30% of children with atopic dermatitis, and allergic rhinitis in 35%[2]. According to the International Study of Asthma and Allergies in Childhood (ISAAC), the prevalence of symptoms of atopic dermatitis in children six or seven years of age during a one-year period varied from less than 2% in Iran and China to approximately 20% in Australia, England, and Scandinavia[1]. The aetiology of atopic dermatitis is complex and involves an interaction between genetic and environmental factors as well as the immune system. The hygiene hypothesis has contributed to the understanding of allergic disease, suggesting that the lack of microbial exposure may increase the risk of atopic disease. A protective effect of microbial exposure on asthma has been often described[3-5]. However, epidemiological evidence supporting the association with atopic dermatitis remains inconsistent[6-8].

Based on the hygiene hypothesis it has been suggested that an altered stimulation of the innate immune system might influence the development of allergic diseases. Activation of the innate immune system is mediated by pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), which are present on immune and epithelial cells and recognize pathogen-associated molecular pattern (PAMPs). Morever, several findings indicate that environmental factors with high level of microbial components may interact with TLR polymorphisms on the development of allergic disease[9]. In a previous study it has been shown that prenatal exposure to farm environment has a protective effect on atopic sensitization in children and leads to an increased expression of innate immunity receptors[10].

Moreover, in humans, polymorphisms in TLRs have been shown to be associated with asthma[11-13]. Recently an association between TLR9 promoter polymorphism and atopic eczema has been reported[14].

Previous studies have suggested that gene expression of receptors of the innate immune system is upregulated by exposure to environment rich in microbial compounds. One study has shown that farmer children have an increased expression of TLR2 and CD14[15]. In addition, several studies have demonstrated that farm lifestyle provides a protective environment against the development of allergic diseases[3,16]. However, the direct correlation between presence of allergic disease in children and TLR or CD14 expression was not

made. Defining if there is a difference of gene expression could allow a better understanding of the determinants of atopic dermatitis.

In this study, as gene expression of innate immunity receptors reflects both genetic and environmental influences, we examined if TLRs and CD14 expression in cord blood samples was associated with the development of atopic dermatitis in the first 2 years of life within a prospective birth cohort, the Protection against Allergy-Study in Rural Environments (PASTURE) study. In addition, we evaluated the prenatal effect of environmental exposures related to the hygiene hypothesis on atopic dermatitis. We further investigated whether TLR polymorphisms or prenatal farm environment or the interaction between them modifies the expression of these receptors.

Method

Study design

The PASTURE study is a prospective birth cohort study involving children from rural areas in 5 European countries (Austria, Finland, France, Germany and Switzerland), designed to evaluate risk factors and preventive factors for atopic diseases. The design of this cohort has been described in detail elsewhere[17]. Briefly, pregnant women were recruited during the third trimester of pregnancy and divided in two groups. Women who lived or worked on family-run farms where any kind of livestock was kept were assigned to the farm group. The reference group was composed of women from the same rural areas not living or working on a farm. The questionnaires developed within the PASTURE study group used questions on various exposures from the International Study of Allergy in Childhood (ISAAC), the Allergy and Endotoxin (ALEX) study, and the Prevention of Allergy Risk factors for Sensitization in children Related to Farming and Anthroposophic Lifestyle (PARSIFAL) study. Questionnaires were administered in interviews or self-administered to the mothers at the end of pregnancy, when the children were 2, 12, 18 and 24 months of age.

Study population

Children from the PASTURE birth cohort with data available on atopic dermatitis between 1 and 2 years of age (n=961). Among these children, 818 have data available on the mRNA analysis in cord blood samples and 863 on TLR polymorphisms.

Definitions

Symptoms of atopic dermatitis are reported by the parents in the questionnaires at 12, 18 and 24 months of age. Atopic dermatitis symptom was considered present, when the child

had once in the first 2 years of life the presence of an itchy rash on at least at one or more of the following locations: face, neck, elbows or behind the knees, joint of hands or feet. Children were labelled having doctor diagnosis atopic dermatitis when the parents reported at least once in the first 2 years of life a doctor diagnosis atopic dermatitis.

Farmer children were defined as children whose parents answered positively to the question 'does your child live on a farm?' and whose family ran the farm. Data on farm exposure, smoking during pregnancy, the mode of delivery, gender, birth weight, gestational age, breastfeeding, and farm milk consumption were obtained from the self-reported questionnaires at the 3rd trimester of pregnancy, at 2 months after birth and at 1 year of age. Maternal atopy was defined as ever had asthma or hay fever, which information were self-reported.

Genotyping of different single nucleotide polymorhisms (SNPs)

Genotyping of different SNPs was performed as described previously[18]. In brief, genomic DNA was extracted from whole blood and a primer extension preamplification (PEP) method was applied to reduce the amount of DNA necessary for the analysis. Next, two polymerase chain (PCR) reactions were performed: First, a standard PCR reaction using forward/reverse primers and dNTPs. Then, after removing of excessive dNTPS by shrimp alkaline phosphatase, a base-specific extension PCR was carried out, using extension primers and mass-modified nucleotides. Reaction solution was dispensed onto a 384-format SpectroChip (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint nanodisenser (Sequenom). A matrix-assisted laser desorption ionization time-of-flight mass spectrometer (MALDI, Bruker) was used for data acquisitions from the SpectroChip. Genotyping calls were made in real time with MassArray RT software (Sequenom). Derived genotype frequencies were compared with the expected allelic population equilibrium based on the Hardy-Weinberg equilibrium test to control for technical genotyping errors. cDNA was amplified by using an iCycler (Bio-Rad, Hercules, Calif) in duplicate, with 18s as a reference gene. Polymorphisms in *TLR5* (n=3) and *TLR9* (n=2) were selected as previously described[19]. These SNPs were as follows: *TLR5/T1845C* (rs5744174), *TLR5/C1173T* (rs5744168), *TLR5/A1774G* (rs2072493), *TLR9/T-2622C* (rs5743836), *TLR9/T-2871C* (rs187084).

Expression of Toll-like receptors and CD14

Total RNA was isolated by using the PaxGene RNA Blood Kit (Qiagen, Hilden, Germany) supplemented with RNase-free DNase (Qiagen). For reverse transcription (RT), 600ng of total RNA in a final volume of 60µl were used and adequate amounts of TaqMan® Reverse Transcription Reagents (Applied Biosystems, Foster City, USA) were added. The running conditions were as follows: 10 min 25°C; 40 min 42°C; 5 min 95°C. All PCR reactions were

analyzed on an ABI Prism 7900 Sequence Detection System (Applied Biosystems) with the following running conditions: 45x (2 min 50°C; 10 min 95°C; 15 sec 95°C; 1 min 60°C). The used predeveloped primer/probes (Applied Biosystems) are listed in Table 1. The data presented are normalized values for the endogenous control (18S rRNA and β2 microglobulin). TLR3 expression was excluded from the analyses as the expression level was less than the detection limit on most of these cord blood samples.

Table 1: Primer/probe assay

Assay ID	Gene
Hs00413978_m1	TLR1
Hs00610101_m1	TLR2
Hs00152933_m1	TLR3
Hs00370853_m1	TLR4
Hs00152825_m1	TLR5
Hs00271977_s1	TLR6
Hs00152971_m1	TLR7
Hs00607866_mH	TLR8
Hs00370913_s1	TLR9
Hs00169122_g1	CD14
Hs99999907_m1	β2 microglobulin
Hs99999901_s1	18S rRNA

Statistical analysis

Data analysis was conducted using SAS software version 9.1 (SAS Institute, Inc., Cary, NC). In this study, both definition of atopic dermatitis, doctor diagnosis and symptom present at least once in the first 2 years of life, were used. Association between mRNA gene expression in cord blood and at 1 year of age and atopic dermatitis (doctor diagnosis and/or symptom) was analysed by logistic regression, crude analysis and adjusted for the potential confounders. Linear regression was performed to analyse the association between polymorphisms in TLR and mRNA gene expression, crude analysis and adjusted for the potential confounders. The association was stratified according to the atopic dermatitis status, in order to see if there is an interaction with genotypes on the gene expression. Associations are presented as exponentiated regression coefficients (geometric means ratios: GMR) for numeric data. The following factors were considered as potential confounders (= extraneous variable that correlates (positively or negatively) with both, the dependent variable and the independent variable): farming status, mother with history of asthma or hay fever, gender, the number of older siblings, smoking during pregnancy and the centre. Heterogeneity among centres was tested by meta-analysis and when the test for heterogeneity was significant, the model was adjusted for centre as a random effect estimate, and if there was no heterogeneity as a fixed effect estimate. Estimates of cumulative incidence of atopic dermatitis in the first 2 years of age were calculated by the Kaplan-Meier method. The Cox proportional hazard model was used to analyze the relation

between mother living and being active on a farm during pregnancy and atopic dermatitis. A p-value below 0.05 was considered to be statistical significant.

Results

General characteristics

The characteristics of the study population showed no difference of the distribution of farmers and non farmers between the 5 centers (Table 2). Among children in the non farmer group, the frequency of parents with allergy and the mothers smoking during pregnancy were higher compared to the children in the farmer group. Among farmer children, the numbers of siblings, breastfeeding at 2 months of age and the consumption of farm milk in the first year of life were higher compared to non-farmer children. For the other characteristics, there were no differences depending on the farming status.

At 1 year of age, the prevalence of doctor diagnosis atopic dermatitis was 10.8% and of atopic dermatitis symptom, 15.1% (data not shown). In the 2 first years of life, the cumulative prevalence of doctor diagnosis atopic dermatitis increased until to 17.9% and of atopic dermatitis symptom, to 25.8%, (Table 2). These cumulative prevalences for symptoms as well as for doctor diagnosis of atopic dermatitis were lower in farmer children compared to non-farmer children. However, the differences were not significant.

Table 2 Characteristics of the study population and prevalence of atopic dermatitis according to farming status

	Total study population		Farmer		Non farmer	
	N=961		N=454		N=505	
	%	(n)	%	(n)	%	(n)
Girl	49.5	(475)	48.9	(222)	50.1	(253)
Centre						
A: (Austria)	20.8	(200)	20.0	(91)	21.6	(109)
C: (Switzerand)	21.3	(205)	20.7	(94)	21.8	(110)
F: (France)	17.4	(167)	17.4	(79)	17.4	(88)
G: (Germany)	22.6	(217)	22.0	(100)	23.2	(117)
S: (Finland)	17.9	(172)	19.9	(90)	16.0	(81)
Parents with atopy history						
None	52.0	(498)	61.5	(279)	43.4	(218)
One parent	39.3	(376)	33.5	(152)	44.6	(224)
Two parents	8.7	(83)	5.0	(23)	12.0	(60)
Mode of delivery:						
Vaginal delivery	82.5	(786)	82.3	(372)	82.6	(414)
Cesarean section	17.5	(167)	17.7	(80)	17.4	(87)
Birth weight						
<2500g	1.5	(14)	1.8	(8)	1.2	(6)
2500-4500g	96.0	(921)	95.2	(432)	96.8	(489)
>4500g	2.5	(24)	3.1	(14)	2.0	(10)
Nb of siblings:						
0	37.5	(360)	27.1	(123)	46.7	(236)
1-2	52.7	(506)	56.4	(256)	49.5	(250)
≥3	9.8	(94)	16.5	(75)	3.8	(19)
Breastfeeding at 2 month						
Yes, only	66.2	(586)	64.4	(268)	67.8	(318)
Yes, not only	15.4	(136)	19.5	(81)	11.7	(55)
No	18.4	(163)	16.1	(67)	20.5	(96)
Farm milk in 1st yr	31.4	(300)	55.4	(251)	9.7	(49)
Mother smoking during pregnancy	12.9	(124)	7.7	(35)	17.4	(88)
Symptoms of Atopic Dermatitis Cumulative prevalence[a]	25.8	(246)	25.2	(114)	26.2	(131)
Dr diagnosis Atopic Dermatitis Cumulative prevalence[a]	17.9	(172)	16.7	(76)	18.8	(95)

[a]Cumulative prevalence from 1 to 2 years of age

Association between mRNA expression and atopic dermatitis

Children with doctor diagnosed atopic dermatitis have a significantly decreased expression of TLR5 and TLR9 in cord blood (adjusted GMR and 95%CI: 0.64, 0.47 to 0.88 and 0.67, 0.51 to 0.90, respectively). The same tendency is observed with TLR1, 2, 4, 6, 7, 8 and CD14 but not statistically significant (Fig. 1A). None of the confounding factors (maternal atopy, gender, siblings, maternal smoking during pregnancy, centre) had a major influence on the results. Among children with atopic dermatitis symptoms, as the outcome, the level of TLR5 and TLR9 expression was also strongly reduced compared to children without symptoms, even though not statistically significant (Fig. 1B). The level of TLRs mRNA was categorised in tertiles and the association with atopic dermatitis was analyzed with the lower category as the reference. The risk to develop atopic dermatitis in the first 2 years of life in children with a level of TLR5, TLR9 or CD14 expression in the upper tertile was reduced by around 50%

(adjusted OR and 95%CI: TLR5: 0.51, 0.32 to 0.81; TLR9: 0.47, 0.30 to 0.75; CD14: 0.56, 0.35 to 0.88) compared to children with an expression in the lower tertile (Table 3).

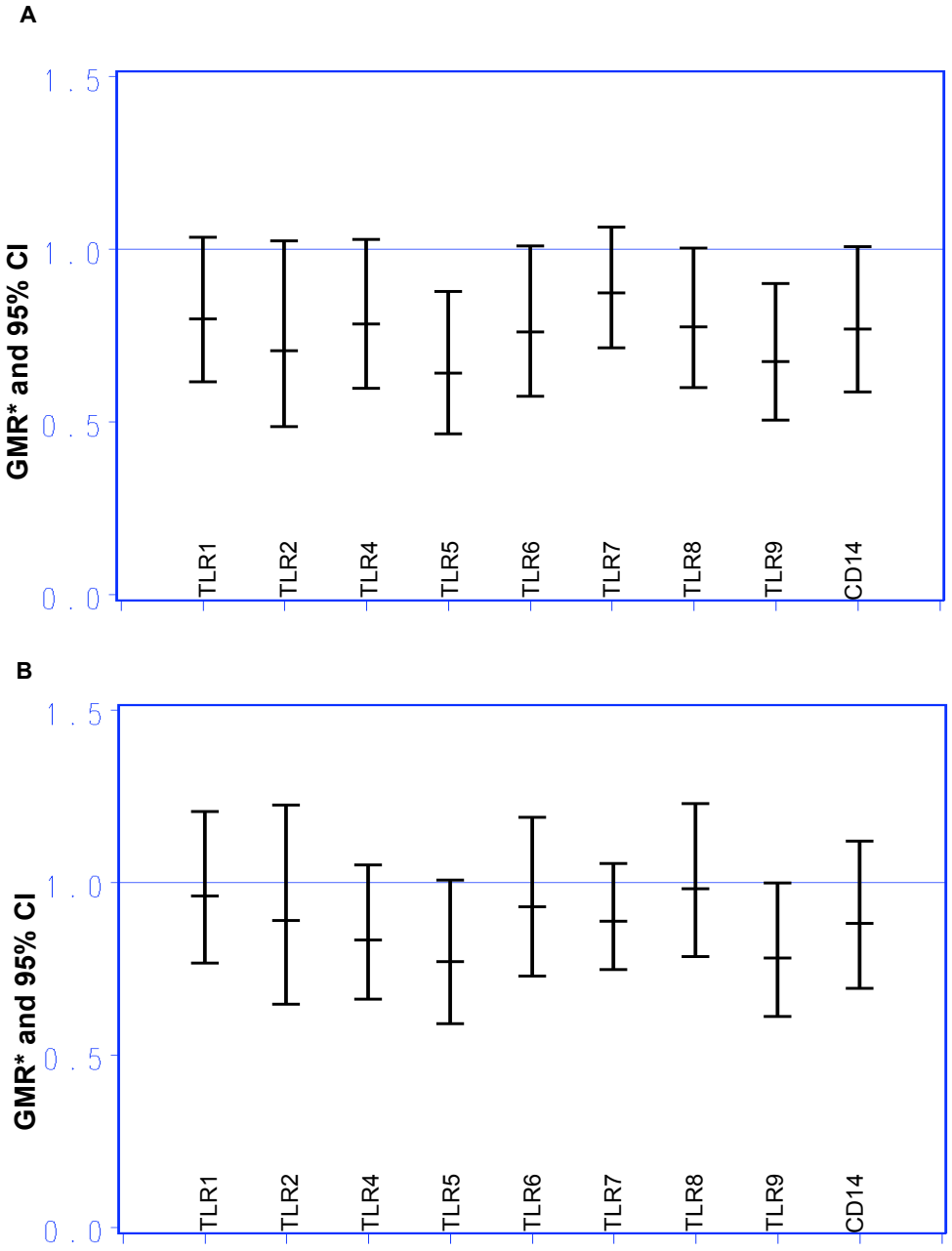


Figure 1. Geometric mean ratio* of TLRs and CD14 expression in cord blood in children with doctor diagnosis of atopic dermatitis (A) and in children with symptoms of atopic dermatitis (B) in the 2 first years of life

*adjusted for farmer, atopic mother, gender, older siblings, smoking during pregnancy and centre

Table 3. Association between TLRs or CD14 expression categorized in tertile and atopic dermatitis

	Doctor diagnosed Atopic Dermatitis	
	OR*	**(CI 95%)**
TLR1		
Upper tertile	0.69	0.44-1.10
Middle tertile	0.78	0.49-1.24
Lower tertile, ref.	1.00	-
TLR2		
Upper tertile	0.66	0.42-1.05
Middle tertile	0.91	0.58-1.42
Lower tertile, ref.	1.00	-
TLR4		
Upper tertile	0.64	0.40-1.03
Middle tertile	1.00	0.64-1.55
Lower tertile, ref.	1.00	-
TLR5		
Upper tertile	**0.51**	0.32-0.81
Middle tertile	0.69	0.45-1.07
Lower tertile, ref.	1.00	-
TLR6		
Upper tertile	0.71	0.45-1.13
Middle tertile	0.91	0.58-1.43
Lower tertile, ref.	1.00	-
TLR7		
Upper tertile	0.69	0.44-1.08
Middle tertile	0.67	0.42-1.05
Lower tertile, ref.	1.00	-
TLR8		
Upper tertile	0.66	0.41-1.06
Middle tertile	1.03	0.67-1.60
Lower tertile, ref.	1.00	-
TLR9		
Upper tertile	**0.47**	0.30-0.75
Middle tertile	**0.61**	0.39-0.95
Lower tertile, ref.	1.00	-
CD14		
Upper tertile	**0.56**	0.35-0.88
Middle tertile	0.66	0.42-1.04
Lower tertile, ref.	1.00	-

*adjusted for farmer, atopic mother, gender, older siblings, smoking during pregnancy and centre

Association between polymorphisms in TLR5 and TLR9 and mRNA gene expression

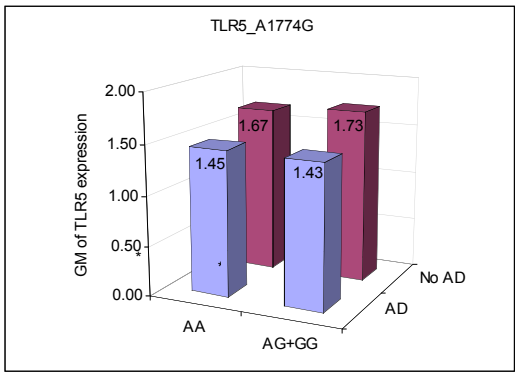
Table 4 shows the genotype frequencies of the 5 investigated TLR5 and TLR9 polymorphisms. Genotype frequencies did not differ between farmer and non farmer children. Mean gene expression of TLR5 was similar in *TLR5/T1845C*, *TLR5/C1173T*, or *TLR5/A1774G* genotypes. There was also no difference of mean gene expression of TLR9 between *TLR9/T-2622C* and *TLR9/T-2871C* genotypes (Fig. 2).

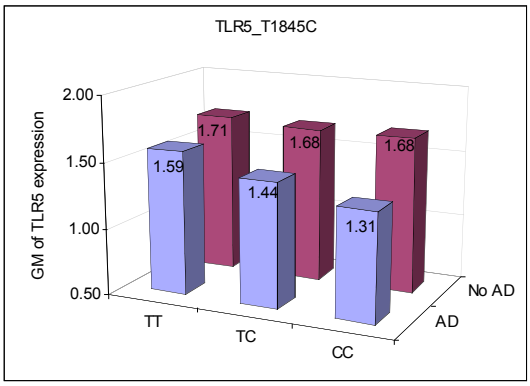
Moreover, the difference of gene expression of TLR5 and TLR9 between children with atopic dermatitis and children with no atopic dermatitis was not modified by these genotypes variants.

	Birth N=863 % (n)	Farmer N=406 % (n)	Non farmer N=457 % (n)
TLR5_T1845C			
TT	32.9 (279)	35.5 (141)	30.5 (138)
TC	49.2 (418)	47.4 (188)	50.9 (230)
CC	17.9 (152)	17.1 (68)	18.6 (84)
TLR5_C1173T			
CC	88.3 (762)	88.4 (359)	88.2 (403)
CT	11.1 (96)	10.6 (43)	11.6 (53)
TT	0.6 (5)	1.0 (4)	0.2 (1)
TLR5_A1774G			
AA	71.8 (607)	73.7 (291)	70.1 (316)
AG	26.7 (226)	25.1 (99)	28.2 (127)
GG	1.5 (13)	1.3 (5)	1.8 (8)
TLR9_TMIN2622C			
TT	75.9 (652)	75.8 (304)	76.0 (348)
TC	22.6 (194)	22.9 (92)	22.3 (102)
CC	1.5 (13)	1.3 (5)	1.8 (8)
TLR9_TMIN2871C			
TT	31.1 (265)	32.9 (131)	29.5 (134)
TC	50.6 (432)	47.2 (188)	53.6 (244)
CC	18.3 (156)	19.9 (79)	16.9 (77)

Table 4. TLR5 and TLR9 polymorphisms. SNPs on different gene positions are depicted in the left column. TT means homozygous for thymine, TC heterozygous for thymine.

A)





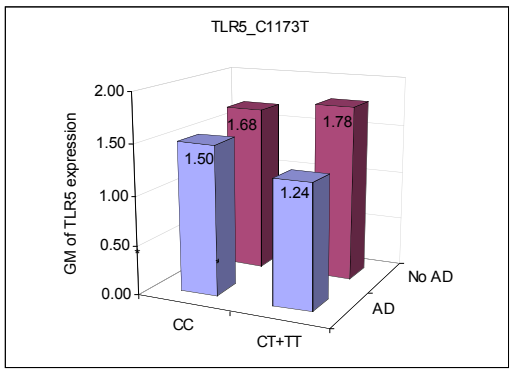
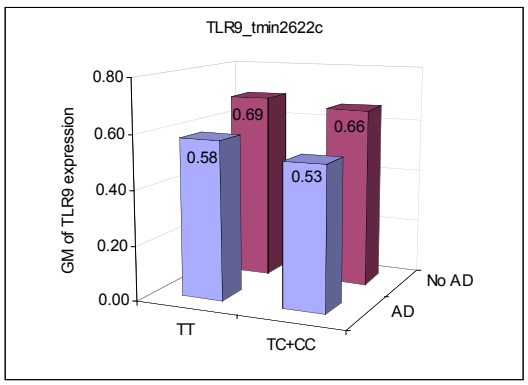
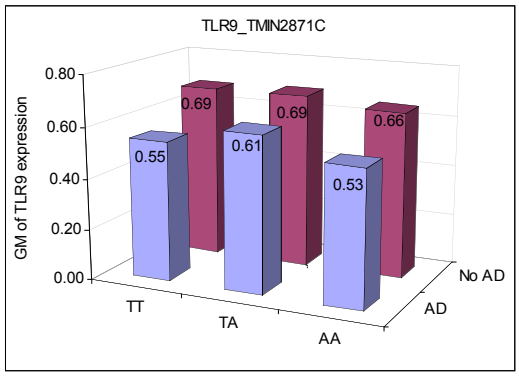


Figure 2. Association between polymorphisms in TLR5 (A) and TLR9 (B) and TLRs gene expression stratified for atopic dermatitis status.

B)

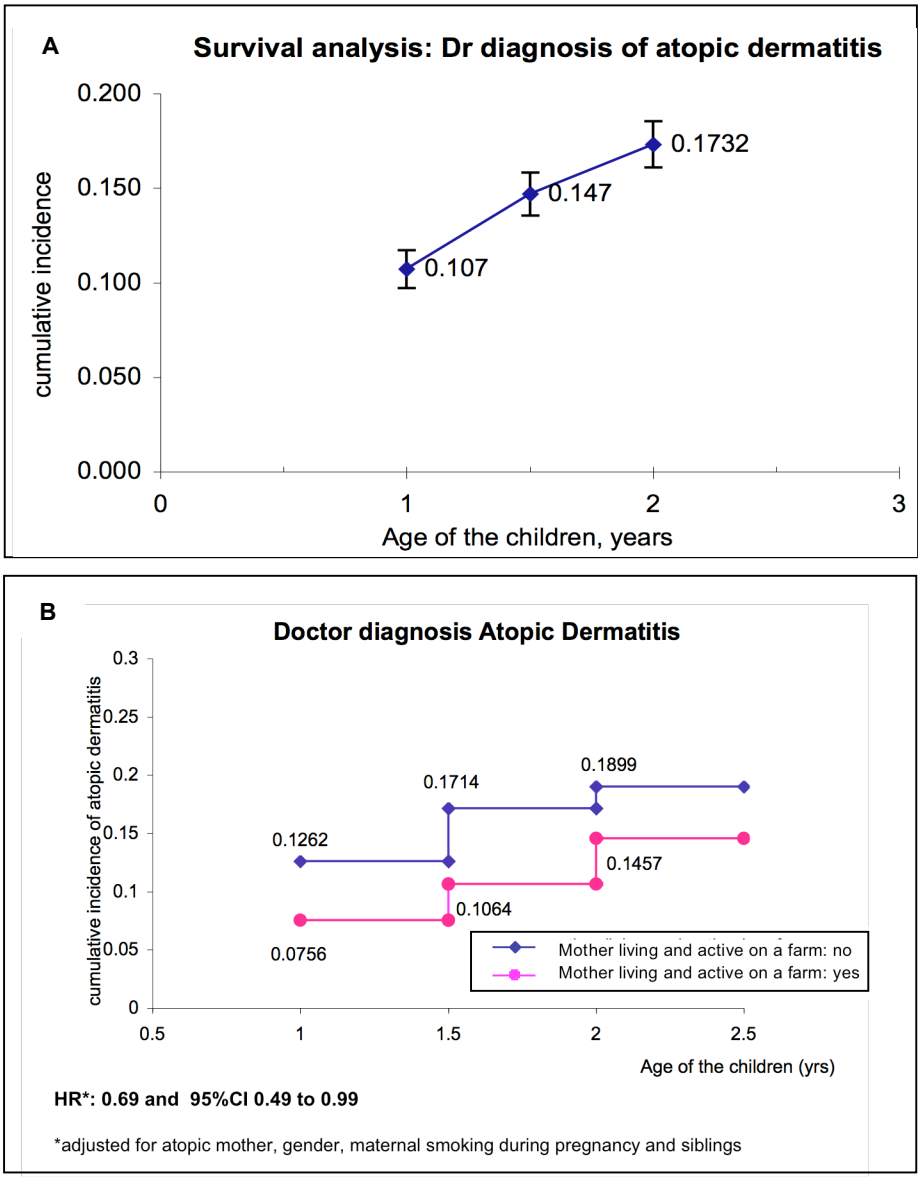




Association between prenatal farm exposure and atopic dermatitis

The cumulative incidence of doctor diagnosed atopic dermatitis at the age of 2 years in the total population was 17.3% (Fig. 3A). Among children with mother living and active on a farm during pregnancy this cumulative incidence was lower compared to children with mother not living and active on a farm during pregnancy (14.6% and 19.0%, respectively and hazard ratio 0.69; 95% CI 0.49 to 0.99, Fig. 3B).

Moreover, we found a significant negative association between the increasing numbers of animal species the mother had contact during pregnancy and the presence of atopic dermatitis in the first 2 years of life (Fig. 3C).



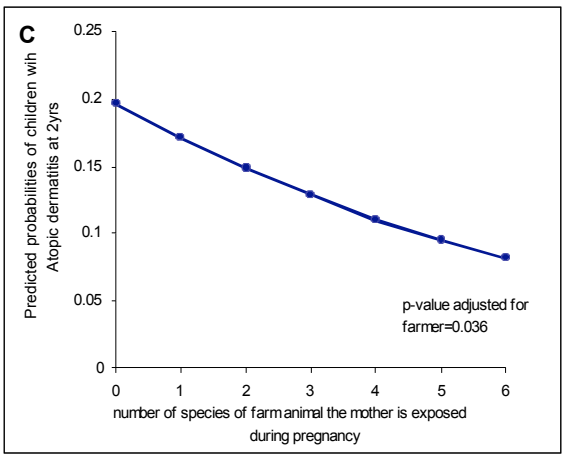


Figure 3. Association between prenatal farm exposure and the development of atopic dermatitis in children in the first 2 years of life.

A: Association between the incidence of doctor diagnosis for atopic dermatitis and age of the children.

B: The incidence of doctor diagnosed atopic dermatitis at age 1, 1.5 and 2, when the mother was living and being active on a farm during pregnancy.

C: Association between the increasing numbers of species the mother had contact during pregnancy and the presence of atopic dermatitis in the first 2 years of life.

Effect of TLR5 and TLR9 genotypes and farming status on gene expression of TLR5 (a) and TLR9 (b) in cord blood

As we have shown before, the gene expression of TLR5 and TLR9 did not varied with genotypes. Moreover the level of TLR5 expression in cord blood was not modified by farming status and the mean gene expression of TLR9 was slightly, but not significantly increased in cord blood of children with mother living on a farm (data not shown). However, stratification by genotypes of *TLR5/A1774G* and *TLR9/T-2622C* revealed heterogeneity of the farm effect on TLR5 and TLR9 expression, respectively. Children with mother living on a farm and having AA genotype in *TLR5/A1774G* expressed 12% more TLR5 in cord blood compared with children with mother not living on a farm. The same significant effect of farming was observed in the expression of TLR9 in children having TT genotype in *TLR9/T-2622C.* In contrast, among children with the AG or GG genotypes in *TLR5/A1774G* and among those with the TC or CC genotypes in *TLR9/T-2622C*, the farm environment did not influence the gene expression (Fig. 4).

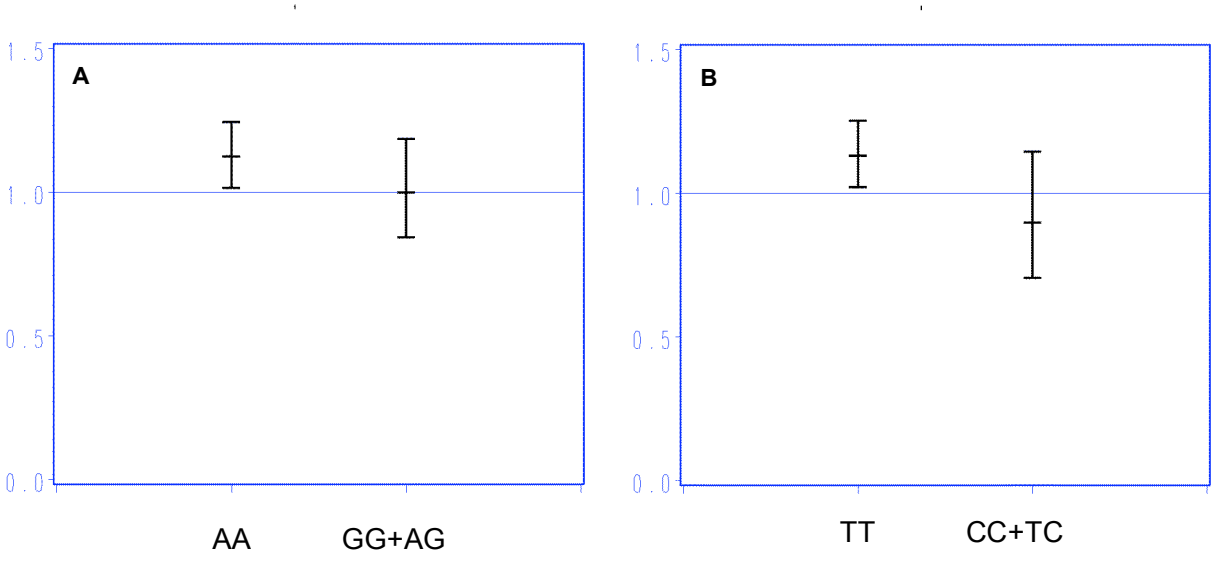


Figure 4. A: Children, with mothers living on a farm and having an AA polymorphism on position 1774 of the TLR5 gene, express more TLR5 compared to children with mothers not living on a farm. GG and AG polymorphisms were combined because of small numbers.

B: Children, with mothers living on a farm and having a TT polymorphism on position 2622 of the TLR9 gene, express more TLR9 compared to children with mothers not living on a farm. CC and TC polymorphisms were combined because of small numbers.

Discussion

This study shows that children with a higher level of TLR5 and TLR9 expression at birth have a decreased risk of atopic dermatitis compared to children with a lower level. Furthermore, maternal farming activities and the contact to different farm animals during pregnancy have a protective effect on the development of atopic dermatitis.

The difference in gene expression of receptors of innate immunity observed at birth, between children who will develop in early life atopic dermatitis and those who will not, suggests an influence of prenatal environmental factors. In fact, it was earlier shown that prenatal exposure to farm environments protects against atopic sensitization[10]. TLRs play a pivotal role in the induction of first line of defense mechanisms of the innate immune system and trigger effective adaptive immune responses to microbial compounds. We have shown in chapter A1 that prenatal and early postnatal farm environmental factors increased the expression of different TLRs at time of birth and at one year of age. Here, we have also observed that genetic variations in combination with farming exposures influence TLR5 and TLR9 gene expression, indicating that a gene-environment interaction may exist. Differences in gene expression between healthy children and such with atopic dermatitis were independent of several single nucleotide polymorphisms (SNPs). However, several SNPs in genes encoding for TLRs have been shown to be associated to allergic diseases, especially with asthma[11-13]. In two previous studies, associations between TLR2 and TLR9 SNPs with atopic dermatitis have been reported[14,20]. But these studies included mainly adult patients and controls and did not investigate the TLR expression levels as well the environmental influences.

The observed differences in TLRs expression concern mainly TLR5 and TLR9. TLR5 recognizes flagelllin[21], found on nearly all motile bacteria and TLR9 recognizes unmethylated CpG motifs present in bacterial DNA[22]. Activation of TLR9 in dendritic cells induces Th1-based immune response[23]. Therefore, synthetic CpG and their receptors are prime candidates for the prevention and treatment of Th2-associated atopic disorders. In this context, the advantage of TLR9 activation with protective effect on Th2 immune responses has been already shown in a murine model and in first clinical trials of TLR9-based immunotherapies[24]. Our study indicates that these new therapeutic strategies with activation of TLR9 might also have a potential efficacy on atopic dermatitis.

Conclusion

Children with higher expression of TLR5 and TLR9 in cord blood have a reduced risk of atopic dermatitis of 50% in the first two years of life, suggesting an in utero effect. We observed a protective effect of prenatal farm environment on the development of atopic dermatits in children. The upregulation of these receptors of the innate immunity might be mediated by the interaction between genotypes and prenatal farm environment.

Contribution to this work

The PASTURE project was planned and is under coordination of E. von Mutius et al.[17]. RNA-isolation, reverse transcription and gene expression measurements were done by myself at the Children's Hospital Zürich. Single nucleotide polymorphisms were analyzed by M. Kabesch at the Children's Hospital (University Munich). Statistical analyses were performed by Caroline Roduit at the Children's Hospital Zürich. The manuscript was written by Caroline Roduit and myself.

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CHAPTER A3

Maternal vitamin D intake during pregnancy increases gene expression of ILT 3 and ILT4 in cord blood

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Abstract

Background

Recent studies indicate that prenatal vitamin D intake protects from the development of atopic diseases in young children. Vitamin D has been shown to induce tolerogenic antigen presenting cells such as dendritic cells. Whether the protective potential of prenatal vitamin D is mediated through tolerance induction, however, is unknown.

Objective

To evaluate the association between prenatal vitamin D supplementation and tolerogenic antigen presenting cells in cord blood as determined by mRNA measurement of immunglobulin-like transcripts (ILT)3 and ILT4

Methods

A prospective multicenter birth cohort was established in rural areas of five European countries. Information on maternal exposures including vitamin D intake was collected by questionnaires during pregnancy. The gene expression of ILT3 and ILT4 was analyzed by real time PCR in the cord blood of the enrolled children.

Results

Maternal vitamin D supplementation during pregnancy was related to an increase in the gene expression of ILT4 ($p=0.003$) and in tendency for ILT3 ($p=0.068$). This association remained significant after adjustment for farming status and gender which were found to be independent factors associated with the gene expression of ILT3 and ILT4.

Conclusions

Vitamin D supplementation during pregnancy increased mRNA levels of ILT3 and ILT4 in cord blood. This may point towards an early induction of tolerogenic immune responses by vitamin D.

Contribution to this work

The PASTURE project was planned and is under coordination of E. von Mutius et al.[17]. RNA-isolation, reverse transcription and gene expression measurements were done by myself at the Children's Hospital Zürich. Statistical analyses were performed by Mascha Rochat at the Children's Hospital (University Munich). The manuscript was written by Mascha Rochat.

CHAPTER A4

Discussion and outlook

The development of allergic diseases such as allergic rhinitis, atopic dermatitis, food allergies and allergic bronchial asthma, which are complex inflammatory disorders, is influenced by two risk factors: On one hand many gene polymorphisms have been identified in the last few years which show an association to one or more manifestations of allergic disorders. On the other hand the rapid increase in the prevalence of allergic diseases in industrialized countries over the last decades is associated with reduced exposure to bacterial, viral or fungal components and changes in subsequent complex gene-environment interactions.

In this work, we proceed with earlier findings from epidemiological studies which have shown that farming factors protect against the development of allergies but also reveal that the time of exposure is crucial. The longitudinal and prospective design of the PASTURE study allowed us to investigate pre and early postnatal factors on the development of the innate immunity as first line of defence against microbial compounds. Indeed, our results provide additional evidence of the importance of early stimulation: Working on a farm during pregnancy affected the expression of different TLRs at time of birth (Chapter A1) and was associated with a reduced incidence to develop atopic dermatitis in the first two years of life (Chapter A2). Early consumption of farm milk, especially unboiled farm milk, influenced the TLR-expression at one year of age (Chapter A1).

What are the farming factors which have the impact to influence TLRs very early in life? It seems likely that the farm area is rich in various microorganisms which are detected by receptors of the innate immunity. Investigation on mattress dust and animal shed dust samples identified a great diversity where *Bacillus* spp and coliform bacteria were the most prominent microorganisms[1]. Higher levels of lipopolysaccharide[2,3] (cell wall components of Gram-negative bacteria), muramic acid[4] (part of cell wall of all bacterial species) and bacterial DNA, rich in hypomethylated CpG motifs[5] have also been identified to be much higher in farming environments, exerting an anti-allergic effect. However, whether one or more bacterial-derived compounds may be directly responsible for the observed allergy-preventing effects cannot be proven in epidemiological analysis. Well-defined experiments, including *in vitro* and *in vivo* laboratory animal studies, are essential to verify the relationship between exposure to certain bacterial-derived substances and the underlying mechanisms, leading to protection against allergic immune responses. First investigations were made by Vogel et al. recently, who studied immune responses, elicited by *Bacillus licheniformis* spores *in vitro* and *in vivo*. This is the predominant *Bacillus* species, which was found in children`s mattresses and animal sheds. Some immunostimulatory activity of *Bacillus* spores could clearly been demonstrated, including an increased Th1-cytokine profile after stimulation of PBMCs and human monocyte-derived dentritic cells and a decreased numbers of eosinophils and reduced mucus-producing goblet cells in a murine allergic asthma model[1].

These results support our findings that there is possibly a way to develop a vaccine-like therapeutic agent for the pre- or early postnatal phase, which has the efficiency to stimulate the innate immunity and protects against allergic diseases. In the past, the association of probiotica supplementation during pregnancy and early infancy and the incidence of allergic rhinitis or asthma during childhood was investigated intensively[6-11]. However, the results are not consistent and the most beneficial probiotic strain or the composition of different probiotics and/or prebiotics have to be determined.

We focused our study about early environmental exposure on the expression of TLRs because these receptors operate as key sensors of invading pathogens in innate immune defenses and are important initiators of subsequent immune responses. Beside TLRs, NOD-like receptors (NLRs), especially NOD1 and NOD2 are known to recognize components of Gram-positive and Gram-negative bacteria[12-14] (see also Introduction). Further analysis with focus on NOD1 and NOD2 gene expression is now carried out for a better understanding of pre- and early postnatal farming exposure influences on the development of innate immune receptors.

Recently, Prescott et al.[15] studied the association of cytokine responses after stimulation of cord blood mononuclear cells with TLR ligands and the diagnosis of allergic diseases at one year of age. Children with allergic symptoms were characterized to have significantly higher TNF-α and IL-6 levels in response to TLR2, TLR4, and TLR5 activation, which might suggest differences in downstream intracellular events that drive cytokine production in subsequently allergic children. The PASTURE study provides the possibilities to investigate in a longitudinal way the expression of members of the signalling cascade, which activate the subsequent cytokine production. However, we have realized that the enormous amount of data and the complexity of the regulatory network of the innate immunity, necessitates suitable alternative methods for data analysis. Popular and interesting tools for gene expression analysis are clustering methods which are used to place data elements into related groups without advance knowledge of the group definitions[16-18]. Further data analyses including such methods have to be realized in the future.

In the past, epidemiological studies contributed to a better understanding of gene-environment interaction and the outcome of immune responses, especially in context of allergic diseases. Such studies provide the advantage to investigate conditions over a period of time, which can hardly be simulated in the lab. A good strategy for investigations and experiments, a close cooperation of different centres, laboratories and fieldworker, as well as sufficient funds, are essential requirements for success. Although the PASTURE-study ran

since 2002, we are only at the beginning of the results that are arising from this prospective study design. Investigation of the maturing innate immune system but also B- and T- cell responses in 4.5 years old children are the following steps, which will contribute to further insights in the complex network of the immune system.

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CHAPTER B1

The use of attenuated Salmonella strains in an ovalbumin (OVA) - induced mouse model of food allergy

Abstract

Attenuated *Salmonella enterica* serovar Typhimurium is an interesting organism for vaccination studies due to the stable and efficient expression of heterologous antigens in the mucosal digestive tract. We hypothesized that attenuated *Salmonella* delivering the model allergen ovalbumin could also be a suitable tool to prevent ovalbumin-induced food allergy in mice. In this study we tested the allergen expression in different attenuated *Salmonella* strains and investigated furthermore the influence of pre-treatment with such bacteria on the development of allergic symptoms in a murine ovalbumin allergy model. Our data indicate that the prophylactical gavage of attenuated *Salmonella* expressing ovalbumin could protect against food-induced allergy.

Introduction

In developed countries, food allergy has increased in the past 10-15 years and up to 8% of children less than 3 years of age and approximately 2% of the adult population suffers on food allergy[1]. The development of new therapeutic or preventive treatments, beside the present method of identification and avoidance of triggering foods, and reactionary therapy in the event of anaphylaxis, is enforced. Although there are various promising results observed, each trial has its limitations. Several attempts of subcutaneous, sublingual or oral immunotherapies, which are partly used successfully for other forms of allergic diseases aim to alter the allergic response to the causal protein without side effects. The advantage of these approaches is that the food can be consumed without symptoms. Although attempts of subcutaneously delivered allergen immunotherapy resulted in a Th1-dominant immune response, significant adverse effects, including recurrent anaphylaxis has been observed[2]. An interesting tool for mucosal immunization is the use of attenuated live bacteria species such as *Salmonella*. Attenuated *Salmonella* are described as a carrier system for the *in vivo* delivery of eukaryotic expression plasmids[3]. SL2707 *aroA* mutant *Salmonella* strain is well characterized in murine immunization studies and was first described as non-virulent and safe carrier strain in 1981[4]. Meanwhile, humoral and cellular immune response of *aroA* mutant *Salmonella* carrier strains in vaccine immunization studies was investigated extensively[5-9] and, recently, first results with the use of *Salmonella* in food allergy studies were published[10]. It was reported that the balance of attenuation of virulence and penetration of the host seems to be an important parameter, since it was observed that carrier strains expressing high levels of heterologous antigens will be over-attenuated and lose their immunogenicity[11,12]. Thus, the interest has increased to generate further attenuated *Salmonella* Typhimurium strains. Strains deleted in *htrA* (high temperature requirement), in

galE (encode UDP-galactose 4-epimerases), *purD* (purine synthesis), or *recA* (necessary for DNA repair) were constructed and described as highly attenuated[13].

The aim of this study was to investigate the expression of a chromosomal or plasmid encoded model allergen (ovalbumin), by different attenuated *Salmonella* strains. Furthermore, we suggest that prophylactical gavage with attenuated *Salmonella* modified genetically to express ovalbumin (OVA) allows protection against OVA-induced food allergy.

Methods:

Bacterial strains and culture conditions

Salmonella Typhimurium strains and plasmids used in this study are listed in Table 1 and 2, respectively. Bacteria were routinely cultured in Luria-Broth (LB, MP Biomedicals), Terrific Broth (TB, QBiogene) and on LB agar (Becton Dickinson) plates. If required for the selection or to maintain plasmids, ampicillin (100 µg/ml) and/or kanamycin (50 µg/ml) were added. All bacterial strains used are attenuated through mutations in genes essential for aromatic amino acid synthesis (aroA), purine biosynthesis (purD), lipopolysaccharid (LPS) synthesis (galE), homologous recombination (recA) or virulence (sseC) and phoN. Bacterial strains were obtained from Dr. Michael Hensel (Erlangen, Germany).

Table 1: Bacterial strains and their characteristics

Strains	Mutation	OVA gene location	Promoter	References/Source
MvP457	purD	chromosomal	constitutive	[14]
MvP464	phoN	chromosomal	constitutive	[14]
MvP478	galE	chromosomal	constitutive	[14]
SL7207	aroA	no		[15]
MvP103	sseC	no		[14]
Mvp455	recA	no		[13]

Table 2: Plasmids and their characteristics

Plasmid	Charakteristic	Promoter	References
p2593	low copy plasmid	intracellular active	[15]
p2631	low copy plasmid	constitutive active	[15]

DNA-Standard methods

Plasmid DNA isolation was performed using either the NucleoSpin Plasmid Kit (Macherey and Nagel) or the QIAGEN Maxi kit. The QIAGEN PCR purification kit was used for the purification of PCR-products, the QIAquick gel extraction kit was used for extraction of DNA-fragments from agarose gel. All steps were performed according to the manufacturer′s protocol. The transfer of plasmid-DNA into different strains of *Salmonella* was mediated by electroporation. Cells were transferred into a chilled electroporation cuvette and shocked with

17kV/cm, 200 Ω and 25µF. For preparation of electrocompetent *Salmonella* strains, 250 µl overnight culture was incubated in 12 ml TB medium until OD_{595} of 2. Cells were centrifuged four times at 6000 g at 4°C and washed with 10 ml cold H_2O. Subsequently cells were resuspended in 200 µl 10% glycine (Sigma) and stored on ice until transformation.

Amplification and sequencing of DNA

To verify the presence of the OVA gene in plasmids, Polymerase-Chain-Reaction (PCR) and sequencing were carried out. The used oligonucleotides are listed in table 3, and were synthesized by Microsynth (Switzerland). PCR for sequencing reactions was done using BigDye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems. In brief, 4 µl of BigDye Reaction Mix and 2 µl of sequencing buffer were added to 10 pmol of the primer and 200-500 ng of the plasmid to be sequenced. Aqua dest. was added to reach 20 µl of reaction volume. After PCR reaction, PCR products were loaded on a Sephadex G-50 column and centrifuged to remove unused dNTPs. Sequences were analyzed using ABI PRISM 310 Genetic Analyzer and ABI PRISM data collection software (Applied Biosystems). Results were evaluated manually by sequence comparison.

Table 3: Oligonucleotides used in this study

Designation	Sequence	Application
Ova FwI	5`-ATT CAA GGA GCT CAA AGT CCA CCA TGC-3`	PCR/Sequencing
Lac-Ova-For-EcoRI	5` GAA TTC GCG GAT TAA CAA TTT CAC AC-3`	PCR/Sequencing
Ova RevI	5`GGG AGG AGA AAC ACA TCT GCC AAA G-3`	PCR/Sequencing
Ova-Rev-SmaI	5`GGG AGG AGA AAC ACA TCT GCC AAA G-3`	PCR/Sequencing

Bacterial infection of macrophages and allergen expression

The murine macrophage cell line RAW264.7 (European Collection of Cell Cultures, UK) was cultured in Dulbecco`s modified Eagle medium (DMEM, GIBCO) containing 10 % heat-inactivated fetal calf serum (FCS, PAA/Omnilab) and 1% Antibiotics/Antimycotics (AB/AM, GIBCO) at 37°C at an atmosphere of 5% CO_2. The cells were suspended in 24-well plates at a density of 5 x 10^4 cells per well.

For the infection of RAW264.7 cells, *Salmonella* strains were grown to stationary phase in LB with the appropriate antibiotic selection. The OD_{600} of the cultures was adjusted with LB to 0.2 and the bacteria were washed once with phosphate-buffered saline (PBS, GIBCO). Bacteria were diluted in cell culture media and added to the cells growing in 24-well culture plates at a multiplicity of infection (MOI) of about 10. To synchronize infection, the plates were centrifuged at 500 rpm for 5 min and incubated for 25 min at 37°C in an atmosphere containing 5 % CO_2. After infection the macrophages were washed three times with PBS and incubated for 3 h in cell culture medium containing 100 µg/ml gentamicin (Sigma). The cells were washed once with PBS and lysed with 0.1% Triton X-100 for 10 min at RT and

centrifuged for 1 min at 1200 rpm. The intracellular expression of OVA was analyzed by Western blot analysis.

Protein analysis and Western blotting

Proteins were separated by SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli[16], using 5% stacking and 10% separating gels.

Proteins were transferred onto nitrocellulose-membrane (Protan BA85, Schleicher & Schüll) by wet blotting procedure, using 10 x transfer buffer (0.27 M Tris, 2 M Glycin, 1% SDS, 20% Methanol). The electrotransfer was performed for 40 min at 0.35 A. The membrane was blocked by incubation in 5% powder milk in TBST over night. OVA was detected by incubation with the primary rabbit anti-OVA antibody (Chemicon) and subsequent incubation with secondary antibody anti rabbit IgG (Goat conjugated to horse-radish-peroxidase (HRP, Amersham Bioscience, diluted in TBST for 1 hour at room temperature (RT). The membrane was washed 3-5 times with TBST after each incubation step. Protein was detected by chemiluminescence using ECL system (Amersham Bioscience).

Mice sensitization to OVA, oral administration of Salmonella and oral challenges

Female C3H/HeJ mice were purchased from Jackson Laboratory (USA). All mice were kept at the Animal Facilities of the University of Geneva School of Medicine under specific pathogen-free conditions. Animals were used 4-6 weeks of age and were fed with standard mice pellets without OVA.

A murine model of food allergy was used as published earlier by Li XM et al[17] and Frossard CP et al[18]. Mice (totally 15 animals, 5 per group) were sensitized orally at days 0, 7, 14, and 21 with 20 mg OVA (Sigma) and 10 µg cholera toxin (List Biological Laboratories) in PBS. For bacterial treatment, *Salmonella* were grown over night until mid-log phase. Bacteria were then harvested by centrifugation (4500 rpm) and resuspended in PBS. 10^9 CFU/mouse in a volume of approximately 200 µl were given on three days prior each oral sensitization step (Fig. 1), using a round-tip stainless steel needle. Two groups of control mice were administered either with PBS or with plasmidless bacteria. On day 28 all mice were challenged by intragastric gavage with 50 mg OVA and observed 30 min until they were sacrificed with CO_2. Anaphylaxis was graded using a symptom score, described by Frossard et al.[18]. Score 0: no symptoms, normal activity; score 1: decreased activity, random scratching, myocloni, reversibility of symptoms within 5 min; score 2: marked decreased reactivity, continuous scratching, abnormal breathing, reversibility of symptoms within 10 min; score 3: low reactivity or absence of reactivity, abnormal breathing, death, or no reversibility of symptoms within 15 min.

The body temperature was measured before and 30 minutes after the challenge with the infrared ear thermometer (Braun, Kronberg, Germany).

All experiments were conducted in accordance with the ethical guidelines of Animal Studies Ethics Committee.

Statistical analysis

Values are presented as the mean ± standard error. The significance of body temperature and symptom score was analyzed by linear regression, using SPSS software.

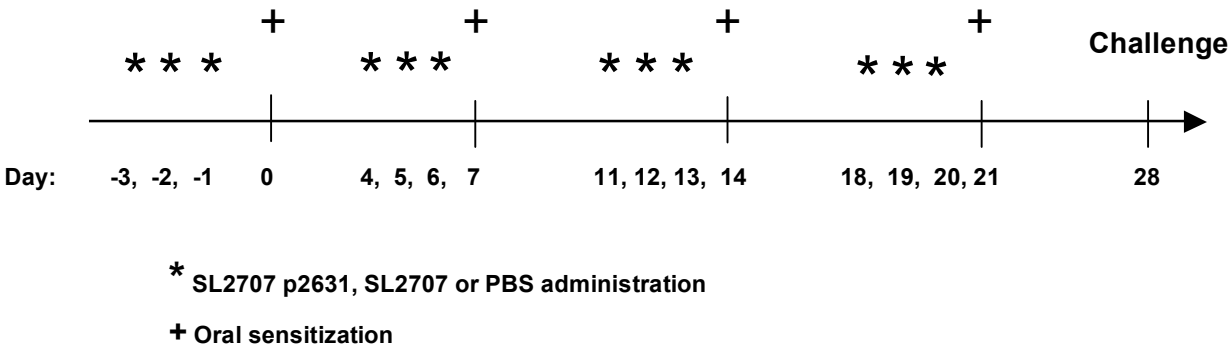


Figure 1. Experimental protocol: Intragastric ovalbumin sensitization and *Salmonella* administration. Mice were sensitized at day 0, 7, 14, 21 with ovalbumin and choleratoxin. Three groups of mice (five animals per group) were fed either with SL2707 p2631, SL2707 or PBS on three days prior each sensitization step and challenged on day 28 with OVA.

Results

Verification of OVA synthesis

Before starting OVA expression analysis the OVA gene was examined by PCR and sequence analysis (data not shown). To verify and to compare the production of OVA, mouse macrophages were infected with different *Salmonella* strains harbouring either OVA encoding plasmids or a chromosomal gene cassette. The intracellular amount of OVA was analyzed by Western blotting (Fig. 2A and B).

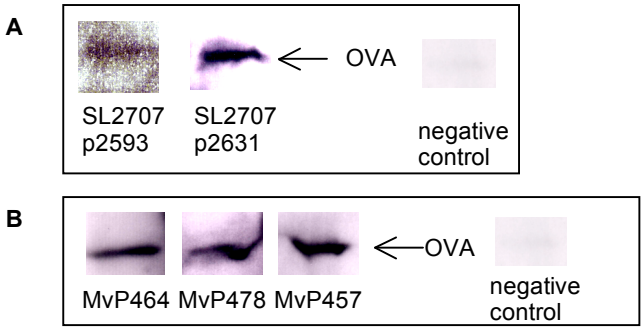


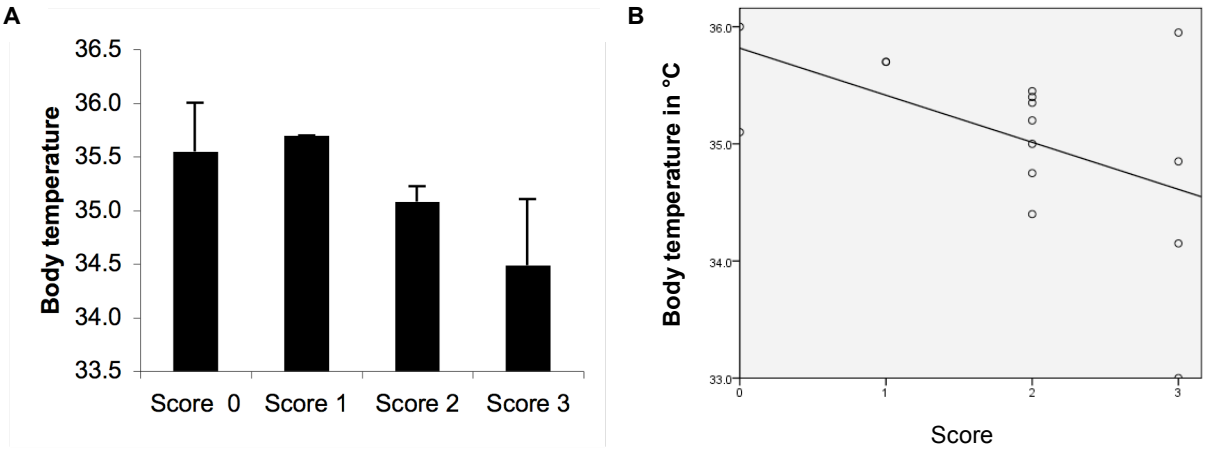
Figure 2. OVA detection *in vitro*. Mouse macrophages were infected with *Salmonella* strains harbouring either OVA-encoding plasmids (A) or expression cassette for OVA (B). The intracellular amount of OVA was analyzed by Western blotting. OVA coded by p2593 is regulated intracellularly and constitutively by p2631. Strains are stable attenuated through mutation in *aroA* (SL2707), galE (MvP464 and MvP478) and purD (MvP457). OVA produced by SL2707 p2593 was only weak detectable. Therefore the brightness and contrast were increased.

OVA was detected clearly in macrophages infected with MvP464, MvP478, MvP457 and SL2707 p2631 but only weak in SL2702 p2593 and not in MvP460. We further transformed p2593 in MvP103 to exclude a strain specific inhibition effect on OVA expression. The observed band was also only weakly visible (data not shown).

Prophylactical treatment of SL2707 p2631

We decided to use the well described *aroA*-deficient *Salmonella* strain harbouring p2631 in a first preliminary *in vivo* sensitization study. The aim of this study was to investigate whether the prophylactical gavage of SL2707 p2631 reduce the severity of allergic reactions. Therefore we administered bacteria to mice on three days before each sensitization step over a period of four weeks. SL2707 without plasmid and PBS served as negative control. After the challenge with high dose of OVA the body temperature was measured before and 30 min after challenge and a symptom score was assessed (Fig. 3A-C). Mice with strong anaphylactic symptoms (score 3) have reduced body temperature (mean: 34.5°C) compared to mice with no (score 0) or weak (score 1) observed anaphylactic symptoms (score 0, mean: 35.6°C; score 1, mean: 35.7; Fig. 3A). The correlation between decrease of the body temperature and increase of the symptom score was slightly over the significance limit (p=0.052; Fig. 3B).

The highest body temperature 30 min after challenge was observed in the group of mice treated with *Salmonella* SL2707 p2631 (mean: 35.5°C) compared to the PBS group (mean: 35.1) and *Salmonella* SL2707 (mean: 34.6°C; Fig. 3C). Furthermore, beside the highest measured temperature in the group of mice treated prophylacticaly with SL2707 p2631, these animals have had reduced anaphylactic symptoms (mean score 1.4), whereas mice treated with PBS or SL2707 have had an identical mean score of 2 (Fig. 3D).



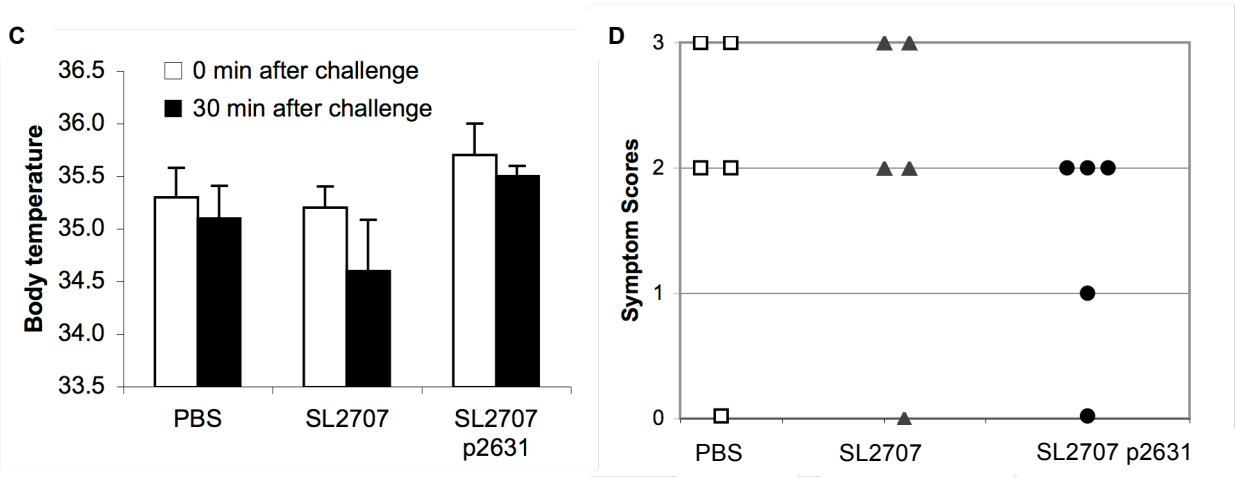


Figure 3. Temperature drop and symptom scores after challenge with OVA.

Mice sensitized to OVA and pretreated with either PBS, SL2707 or SL2707 p2631 were challenged with high dose of OVA and observed for 30 min. The observed anaphylactic symptoms were classified in a score system: Score 0: no symptoms, normal activity; score 1: decreased activity, random scratching, myocloni, reversibility of symptoms within 5 min; score 2: marked decreased reactivity, continuous scratching, abnormal breathing, reversibility of symptoms within 10 min; score 3: low reactivity or absence of reactivity, abnormal breathing, death, or no reversibility of symptoms within 15 min.

A and B: Correlation between body temperature decrease and symptom score increase. The body temperature of each mouse was measured in the ear twice per mouse. Results in A are presented as means ± standard error. For calculation of the p value, a linear regression was done (B).

C: Body temperature as mean values ± standard error per mouse group before and 30 min after challenge.

D: The observed symptoms 30 min after challenge are graded in a score system. Each point corresponds to one mouse.

Discussion and outlook:

In the current study, we investigated first the expression of OVA, encoded either chromosomally or on plasmids and furthermore we tested the prophylactical gavage of one attenuated *Salmonella* strain in an *in vivo* experiment. We were able to verify OVA in nearly all used constructs. The choice of SL2707 p2631 for use *in vivo* experiment was based on the two following reasons: Although it is already known that chromosomal integration provides more stable expression of heterologous antigens[13] such a strain might deliver lower amounts of heterologous antigens due to the single gene copy compared to plasmid-based constructs with multiple copies. Furthermore, we observed that the plasmid-based antigen delivery depends on the promoter. This was already earlier described by Husseiny et al. who observed a reduced OVA expression under control of the intracellular-activated promoter sseA in an *aroA* carrier strain[15].

Results of our *in vivo* experiments indicated that mice pre-treated with SL2707 p2631 may have a positive and protective effect on allergen induced anaphylaxis, characterized through lower mean score and higher body temperature compared to the controls. However, this

preliminary experiment was carried out with a small number of animals and did not allow wide molecular investigation. Therefore, a new *in vivo* experiment was planned (chapter B2) to provide not only insight into immunological processes but also investigate if the use of attenuated *Salmonella* delivering an allergen is also a suitable tool to treat food allergy. Some modifications were carried out for the used construct: Recently a novel combination of heterologous antigens fused on effector proteins of bacterial type III secretory system was described for a vaccination strategy[12]. Effector proteins are bacterial virulence proteins characterized to be transported from the bacteria cell to the host cell cytoplasm[19,20]. We hypothesized a further improvement of the immune response if the allergen is transported directly into the host cell cytoplasm.

Since it was reported that *aroA* deficient strains were shown to be reduced in the ability of protein synthesis, probably due to the defect in the synthesis of aromatic amino acid, we decided to use a *recA* mutant strain for the following *in vivo* experiment, which was already described as avirulent and sensitive to innate immune defense mechanisms[21].

Contribution to this work

In vitro experiments were designed, performed, and analyzed by myself at the lab of R. Lauener at the Childrens's Hospital Zürich. The *in vivo* experiment was planned by myself and carried out together with C. Frossard at the School of Medicine (University of Geneva). The manuscript was written by myself.

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CHAPTER B2

Salmonella enterica serovar Typhimurium as a vector against food allergy

Written as manuscript for publication in Allergy

Johanna Wohlgensinger, Remo Frei, Nathalie Frei, Susanne Loeliger, Caroline Roduit, Roger Lauener

Abstract

Background

The strict avoidance of allergenic substances is the common treatment of food allergy. Allergen-specific immune therapy, where patients are treated with increasing concentrations of the allergen, is a well established method against other allergies, such as hay fever or insect sting allergies but not food allergy. Further, exposure to microbial components protects against the development of allergic disorders. In this study, we tested whether combined intake of microorganisms and low amounts of allergen is a possible method to prevent or treat food allergies.

Methods

An attenuated *Salmonella* strain was transfected with a low copy plasmid encoding the gene for the model allergen ovalbumin (OVA) fused on the effector protein sseF (p2629) or with a control plasmid (p2096).

Mice were administered *Salmonella* p2096, *Salmonella* p2629 or PBS prior to oral sensitization with OVA in combination with choleratoxin (prophylactical approach) or after sensitization phase (therapeutical approach). A symptom score was generated after oral antigen challenge. Allergen-specific antibody titres were measured in serum and feces. Levels of T-helper cell populations as well as relative gene expression of different Toll-like receptors (TLRs) were determined in lymphnodes.

Results

We showed that *Salmonella* releasing allergen reduced allergic symptoms in mice applied in a prophylactic or therapeutic way, along with increased antigen-specific immunoglobulin A (IgA) titres and reduced gene expression of TLRs.

Conclusion

These results suggest that the combination of microbial stimulation and application of small amounts of allergens via intracellular living microorganisms bioengineered to deliver antigen, can decrease food-induced anaphylaxis.

Introduction

Up to 8% of children less than 3 years of age and approximately 2% of adults suffer from clinical symptoms of food allergy. The marked increase of allergic diseases in westernized countries is associated with wealth, good education and small families as well as reduction in infectious diseases. These observations have given rise to the formulation of the ’hygiene

hypothesis', which suggests that limited exposure to bacterial and viral pathogens during early life leads to an insufficient stimulation of Th1-cells as well as a reduced activation of T-regulatory (Treg) cells and a shift to Th2-biased immune responses, resulting in a predisposition to allergy[1-4]. Results of previous cross-sectional studies with farming and non farming children have shown that continuous exposure of the immune system to microbes or its components, such as lipopolysaccharides (LPS) may protect against allergic symptoms[5,6]. There is no treatment of food allergy, except the strict avoidance of trigger foods and the treatment of allergic reactions. Subcutaneously delivered allergen immunotherapy is a well established method to treat insect sting allergies or hay fever but not food allergies[7].

Salmonella enterica serovars Typhimurium is a rod-shaped gram-negative bacterium, characterized to immigrate and live within cells of the intestine system. The ability of *S. enterica* to enter non-phagocytic cells and survive and replicate intracellularly in phagocytes is based on virulence factors encoded by pathogenicity islands (SPI). Two pathogenicity islands (SPI1 and SPI2) encode a type III secretion system (T3SS), which is evolutionarily related to the flagellar export system and mediates the transfer of bacterial virulence proteins, known as effector proteins, from the bacterial cell into the host-cell cytoplasm[8,9]. Many different effector proteins are translocated by the SPI2-T3SS, among them SseF[10].

Attenuated *Salmonella* are attractive 'live vectors' and an interesting tool for mucosal immunization because this delivering system targets the mucosal-associated lymphoid system and brings the expression plasmid in contact with immune cells.

It was described that *Salmonella* strains are unstable when harbouring high copy number plasmids[11]. We have used the low copy plasmid pWSK29 which encodes ovalbumin (OVA) as a model allergen, fused to the effector protein SseF under control of promoter sseA which is activated by *Salmonella* after uptake by dentritic cells (DCs)[12]. The aim of this study was to evaluate whether the oral gavage of attenuated *Salmonella enterica* serovar Typhimurium, expressing OVA-SseF fusion protein, could modulate allergic symptoms prophylactically as well as therapeutically in an OVA-induced murine allergy model. Furthermore, we measured antibody titres, T-helper cell differentiation and the expression receptors of the innate immunity to assess the influence of *Salmonella* on the immune response.

Methods

Bacterial strains and culture conditions

Salmonella strain MvP455 and plasmids p2096 and p2629 used in this study were obtained from Dr. Michael Hensel (Erlangen, Germany) and are described in[13-16]. MvP455 harbours a mutation of *recA* through stable integration of a GFP/Kanamycin gene cassette which is regulated intracellularly by promotor sseA. *recA* mutants have been described as avirulent

and sensitive to the oxidative burst of macrophages[17]. Plasmids p2096 and p2629 are low copy number plasmids that contain either effector protein sseF fused on OVA protein (p2629) or on sseG (p2096) as a control (Fig.1). Bacteria were routinely cultured in Luria-Broth (LB, MP Biomedicals) and on LB agar (Becton Dickinson) plates containing 50 µg/ml kanamycin. For the selection or to maintain plasmids, ampicillin (100 µg/ml) was added.

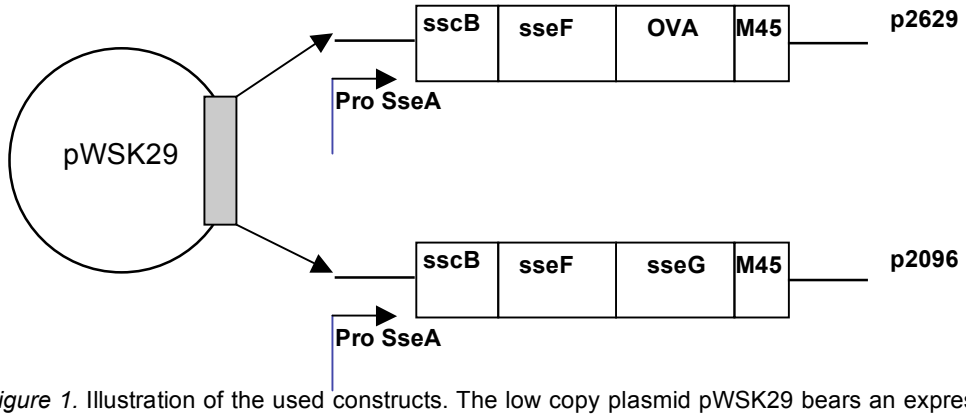

Figure 1. Illustration of the used constructs. The low copy plasmid pWSK29 bears an expression cassette which consists of the *in vivo* activated promoter Pro SseA, the effector protein SseF with the chaperon sscB and a gene fragment encoding either the model allergen OVA (p2629) or the effector protein sseG (p2096).

Bacterial infection of macrophages and allergen expression

The murine macrophage cell line RAW264.7 (European Collection of Cell Cultures, UK) was cultured in Dulbecco`s modified Eagle medium (DMEM, GIBCO) containing 10 % heat-inactivated fetal calf serum (FCS, PAA/Omnilab) and 1% Antibiotics/Antimycotics (AB/AM, GIBCO) at 37°C in an atmosphere of 5% CO_2. The cells were suspended in 24-well plates for experiments at a density of 5 x 10^4 cells per well. The infection of RAW264.7 cells with *S. typhimurium* strains was carried out as described in[18]. The intracellular amount of OVA was analyzed by Western blot analysis. For OVA detection rabbit anti-OVA primary antibody (Chemicon) and goat anti-rabbit IgG (HRP, Amersham Bioscience) as secondary antibody was used. OVA was detected by chemiluminescence using ECL system (Amersham Bioscience).

Animal keeping and mice sensitization

Female C3H/HeJ mice were purchased from Jackson Laboratory (USA). All mice were kept at the Institut für Labortierkunde of University hospital Zurich under specific pathogen-free conditions. Animals were used 4-6 weeks of age and were fed with standard mice pellets without OVA. All experiments were conducted in accordance with the ethical guidelines of Animal Studies Ethics Committee.

A murine model of food allergy as published earlier by Li XM et al[19] and Frossard CP et al[20] was used in the experiments. Mice were sensitized orally at days 0, 7, 14, and 21 with 20 mg OVA (Sigma) and 10 µg cholera toxin (List Biological Laboratories) in PBS. For bacterial treatment, *Salmonella* were grown over night until mid-log phase. Bacteria were then harvested by centrifugation (4500 rpm) and resuspended in PBS. 10^9 CFU/mouse in a volume of approximately 200 µl were given prior each oral sensitization (prophylactical approach, Fig. 2a) or after sensitization phase (therapeutical approach, Fig. 2b), using a round-tip stainless steel needle. The actual infection dose was always verified by serial dilution and subsequent plating on LB-agar plates in the presence of appropriate antibiotic. Two groups of control mice were administered either with PBS or with plasmidless bacteria.

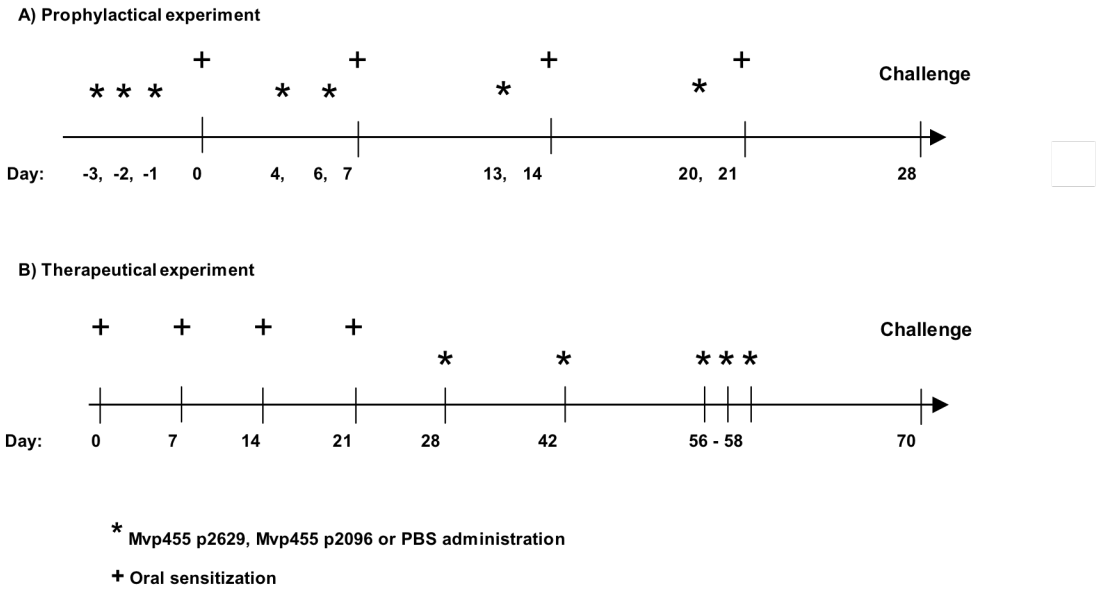

Figure 2. Experimental protocol: Intragastric OVA sensitization and *Salmonella* administration. Mice were sensitized at day 0, 7, 14, 21 with OVA and choleratoxin. Three groups of mice were fed either with Mvp455 p2629, Mvp455 p2096 or PBS prior each sensitization step (Fig. 2A) or after the sensitization phase (Fig. 2B). Mice were challenged with 50 mg OVA at day 28 and 70, respectively.

On day 28 (prophylactical approach) and day 70 (therapeutical approach), respectively, all mice were challenged by intragastric gavage with 50 mg OVA and observed 30 min until they were sacrificed with CO_2. Anaphylaxis was graded using a symptom score. Score 0: no symptoms; score 1: low activity, randomly scratching, reduction of the symptoms within 5 min; score 2: considerable low activity, constantly scratching, abnormal breathing, reduction of the symptoms within 10 min; score 3: considerable low activity, constantly scratching, abnormal breathing, swollen nose, duration of the symptoms > than 10 min; score 4: absence of reaction, death. The scoring of the symptoms was performed in a blind manner as two individuals unaware of sample identities evaluated scores.

Feces preparation

Fecal samples were collected at day 2, 20, 27, 29 (prophylactical approach) and day 0, 16, 30, 58, 60, 70 (therapeutical approach). Extracts of fecal pellets were prepared as described by Marinaro et al.[21]. In brief, 100 mg of fecal pellets was mixed with 1 ml of PBS containing 0.1% NaN_3 and incubated for a minimum of 2 hours at 4°C. Then the pellet was vortexed for 10 min. After centrifugation (4000g, 20 min), supernatants were collected and stored at -80°C.

OVA-specific immunoglobulin measurement

We measured levels of OVA-specific IgE, IgG1 and IgG2 in sera after challenge and IgA in fecal extracts at different timepoints, using an enzyme-linked immunosorbent assay (ELISA). For the assays, plates were coated with 100 µg/ml OVA in coating buffer (pH 9.5) overnight at 4°C. After blocking with PBS + 10% FCS for 1h, 100 µl of samples were added to the plates. Sera were diluted 1:20 for IgE and IgA, 1:100 for IgG3, 1:1000 for IgG2 or 1:5000 for IgG1. Plates were incubated overnight at 4°C. After washing, biotin-conjugated rat anti-mouse IgG1, IgG2, IgE, or IgA monoclonal antibodies (2 µg/ml; BD Pharmingen) were added to the plates for detection of OVA specific immunoglobulin and incubated for 1h at room temperature. Streptavidin HRP (BD Pharmingen) was incubated for 30 min as detecting reagent (1:1000). The reactions were developed with the TMB substrate (BD OptEIA) and stopped with 50 µl 2N H_2SO_4. The plates were read at 450nm.

Tissue homogenization and gene expression measurement

We transferred 30 mg of the frozen lymph nodes to 350 µl RLT buffer (QIAmp RNA Blood Mini Kit, Qiagen) supplemented with 1% β – mercaptoethanol and homogenized it using a Retsch MM300 mixer mill (Retsch). For total RNA extraction, we used the QIAmp RNA Blood Mini Kit (Qiagen) supplemented with RNase-free DNase (Qiagen). For reverse transcription (RT), we used 300 ng of total RNA in a final volume of 30 µl and added adequate amounts of TaqMan Reverse Transcription Reagents (Applied Biosystems). We performed quantitative real-time PCR with 3 µl of RT solution in a final volume of 25 µl and analyzed it with an ABI Prism 7700 Sequence Detection System (Applied Biosystems). All primers were ordered as pre-developed assays from Applied Biosystems. The gene expression values were normalized to the endogenous control 18s rRNA and relative mRNA amounts were determined using the comparative Ct (threshold cycle) method according to the manufacturer's instruction (Applied Biosystems).

Isolation of lymphnode cells and T helper cell determination

Freshly removed lymphnodes were pooled per group in precooled PBS containing 2% FCS (PAA) and kept on ice. The organs were homogenized using a plunger of a 5 ml syringe and

a prewet cell strainer with a 100 µm-mesh (Falcon). The cell suspension was centrifuged at 1400 rpm (10 min at 4°C). To remove the red blood cells, 5 ml sterile lysis buffer (0.15M NH_4Cl, 10mM $KHCO_3$, 0.1M NA_2EDTA, pH 7.2-7.4) was added and the cell solution was incubated for 5 min at 4°C. After centrifugation (1400 rpm, 10 min), the cells were once washed with PBS + 2% FCS and then diluted in cell culture medium (RPMI1640, GIBCO + 10% FCS + 50 µg/ml gentamicin). To determine the T helper cell populations (Th1, Th2, Treg) in lymphocytes, intracellular staining was carried out, using the mouse Th1/Th2 flow panel and the mouse regulatory T cell staining kit (both eBioscience) according to the manufacturer's protocol. The FACS analysis was done in the flow cytometer FACSCalibur (Becton Dickinson). As a control for unspecific binding, isotype controls were used: rat IgG2a for FITC and PE or rat IgG1 for APC (eBioscience). Isotype coated cells were utilized for the settings of the FACSCalibur.

Results

Verification of OVA synthesis

Prior to the *in vivo* experiments, mouse macrophages were infected with *Salmonella* strain MvP455 harbouring either OVA encoding plasmid p2629 (*Salmonella* OVA^+) or the control plasmid p2096 (*Salmonella* OVA^-) to verify the OVA production. Western Blot analysis have shown a clear amount of intracellular OVA (Figure 3 and [16]).

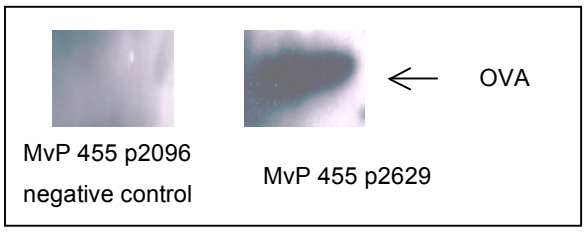

Figure 3. OVA detection *in vitro*. Mouse macrophages were infected with *Salmonella* strain MvP455 harbouring either OVA-encoding plasmids p2629 or control plasmid p2096. The intracellular amount of OVA was analyzed by Western blotting.

Oral challenge to OVA

To investigate whether *Salmonella* strain MvP455 p2629 expressing OVA reduce the severity of allergic reactions, we administered bacteria orally to mice before each sensitization step (prophylactical approach, Fig. 2A) or after sensitization phase (therapeutical approach, Fig. 2B) and challenged them with a high dose of OVA. In the prophylactical approach the assessed symptom score showed no differences between the groups PBS and p2096, which both had a mean score of 2, but a reduced mean score of 0.9 for p2629 (Fig. 4A). Also for the therapeutical approach, mice treated with *Salmonella* p2629 show the least allergic symptoms (mean: 0.8) compared to the control groups (PBS mean: 1.3; p2096 mean: 2; Fig. 4B).

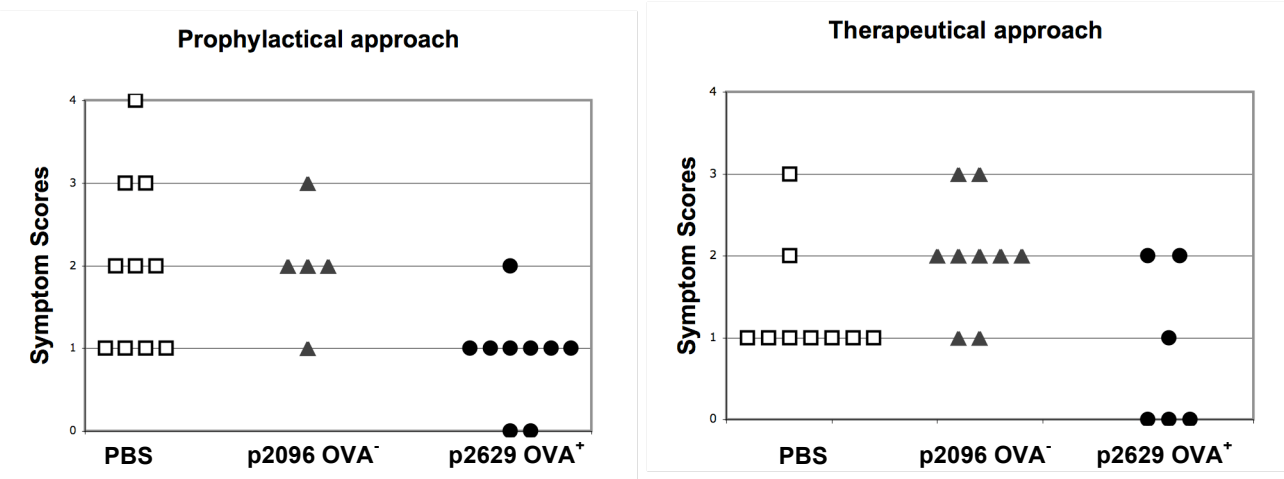

Figure 4. Assessed symptom scores after challenge with OVA. Each square/triangle/point corresponds to one mouse. Scoring system: Score 0: no symptoms; score 1: low activity, randomly scratching, reduction of the symptoms within 5 min; score 2: considerable low activity, constantly scratching, abnormal breathing, reduction of the symptoms within 10 min; score 3: considerable low activity, constantly scratching, abnormal breathing, swollen nose, duration of the symptoms > than 10 min; score 4: absence of reaction, death.

OVA-specific mucosal IgA in feces

To investigate the OVA-specific IgA levels in the gut, feces samples were collected at different timepoints and pooled per mouse group for further OVA-specific IgA analysis. The OVA-specific IgA levels in mice treated prophylactically with *Salmonella* or PBS increased over time (Fig. 5A). However, in both approaches mice treated with *Salmonella* expressing OVA had increased levels of OVA-specific IgA at day of challenge (prophylactical approach OD 1.51 ; therapeutical approach OD: 1.1; Fig. 5A, B) compared to that of the controls (prophylactical approach: PBS OD 0.96; p2096 OD 1.27; therapeutical approach: PBS OD 0.37; p2096 OD 0.67, respectively).

Levels of OVA-specific IgG1, IgG2a/b and IgE in sera

For the measurement of OVA-specific IgG1, IgG2a/b and IgE, serum of mice was pooled per group after challenge. The levels of OVA-specific IgG1 and IgG2a/b in the group of mice treated with *Salmonella* expressing OVA were higher in the prophylactical approach (Fig. 5C, E) compared to the control groups, but not in the therapeutical approach (Fig. 5D, F). By contrast, in the therapeutical approach mice treated with *Salmonella* OVA^+ showed reduced OVA-specific IgE levels (Fig. 5G, H).

Levels of T-helper cell population in lymphnodes

To determine the T-helper cell populations (Th1, Th2, and Treg) in lymphnodes, lymphnode cells were pooled per mouse group and stained for IL-4, IL-2 and IFN-γ or CD4, CD25, FOXP3, respectively, and analysed by flow cytometry. In the prophylactical approach, mice

treated with *Salmonella* expressing OVA showed reduced levels of IL-4, IL-2 and IFN-γ positive cells compared to the control groups (Fig. 6A-C). The levels of $CD4^+CD25^+FOXP3^+$ positive cells were higher compared to the PBS group and slightly higher compared to p2096 (Fig. 6D). In the therapeutical approach, the amount of IL-2 and IL-4 positive cells was higher in both *Salmonella* treated groups (p2629 and p2096) compared to the PBS group (Fig. 6E, F). There was no difference in the PBS group and the mice treated with *Salmonella* expressing OVA for $CD4^+CD25^+FOXP3^+$ positive cells, whereas a decreased amount was observed for p2096 (Fig. 6G).

Expression of Toll-like receptors in cells of lymphnodes

The relative gene expression of different TLRs is presented in Figure 7A (prophylactical approach) and 7B (therapeutical approach). The group *Salmonella* p2629 (OVA^+) showed a ≥ 0.5-fold decrease for TLR2 and TLR8 (prophylactical approach) and TLR2, TLR7, TLR9 (therapeutical approach) compared to the group *Salmonella* p2096 (OVA^-) and PBS. TLR7 (prophylactical approach) and TLR8 (therapeutical approach), respectively was only slightly reduced. CD14 was as well reduced in mice treated with *Salmonella* expressing OVA, however, only in the prophylactical approach. No differences were observed for TLR5 (data not shown).

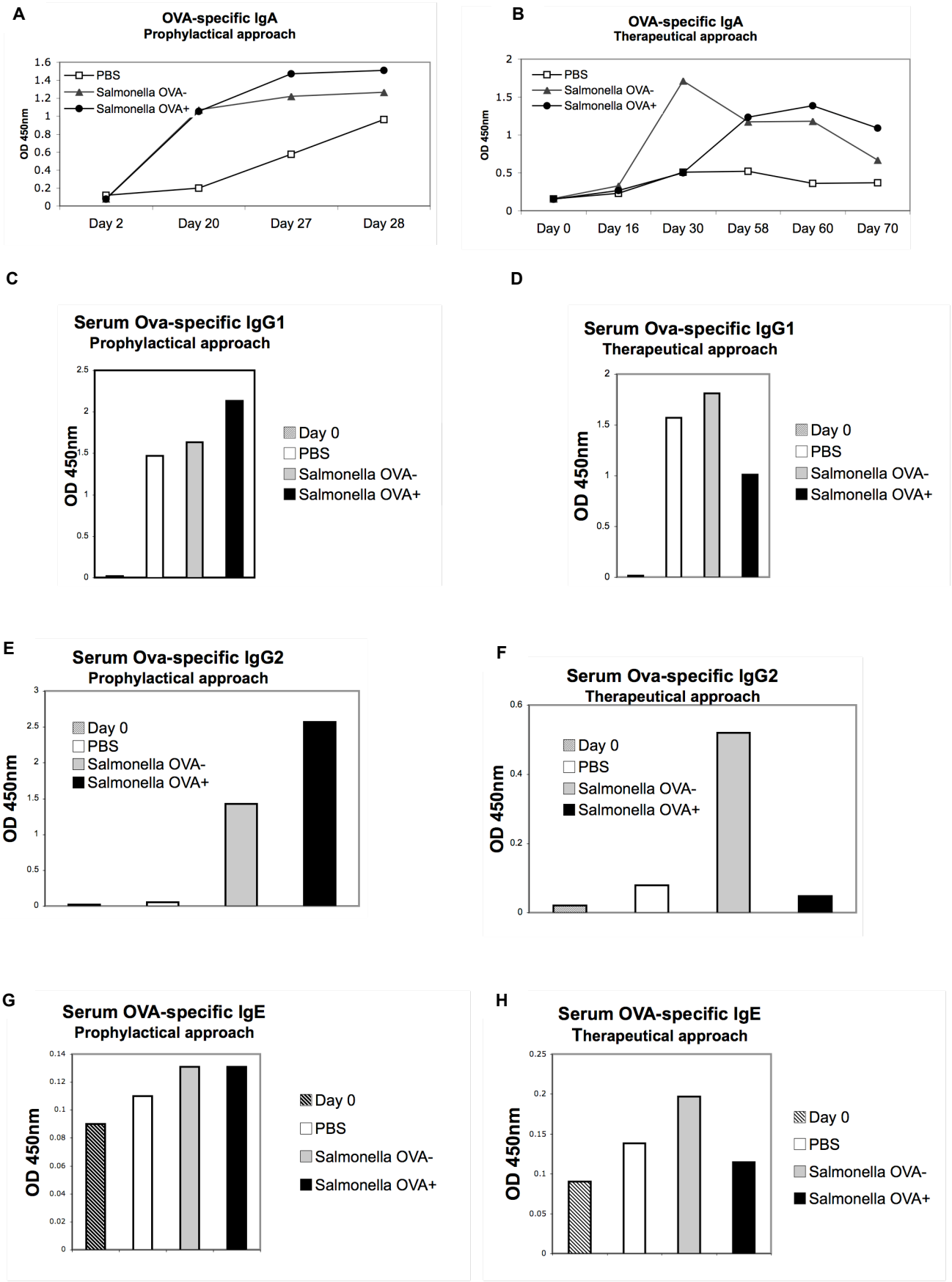

Figure 5. Antibody titres were measured in pooled sera and feces of each mouse group by enzyme-linked immunosorbent assay and presented as optical density. Serum was collected after challenge at day 28 (prophylactical approach) and 70 (therapeutical approach), respectively. Feces was collected at different timepoints during the experiment.

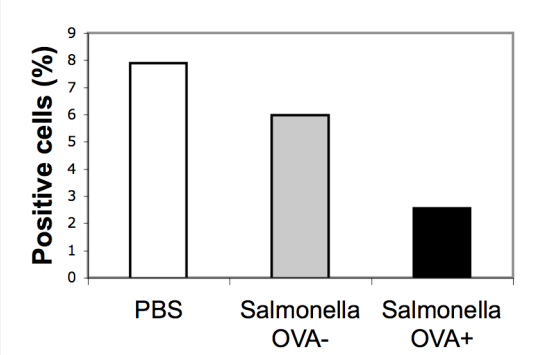

Fig. 6A. IFN-γ positive cells

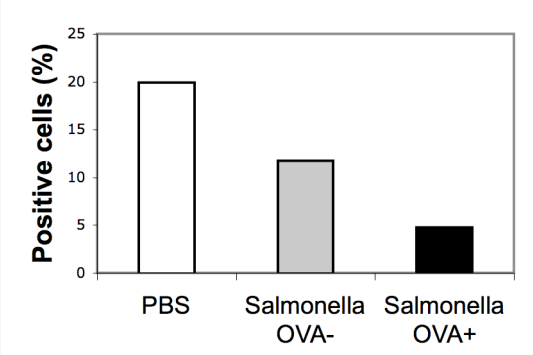

Fig. 6B. IL-2 positive cells

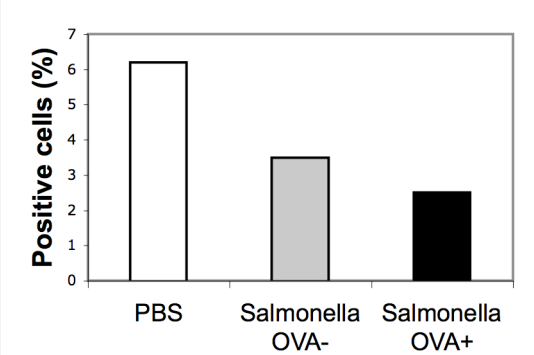

Fig. 6C. IL-4 positive cells

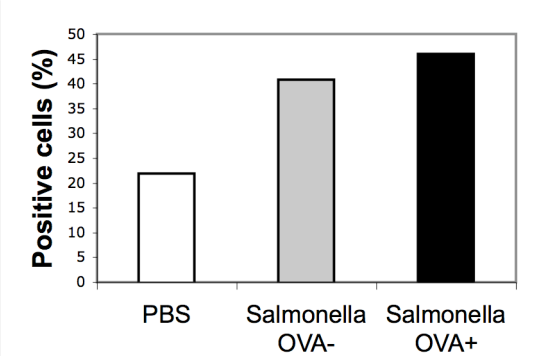

Fig. 6D. FoxP3 positive cells

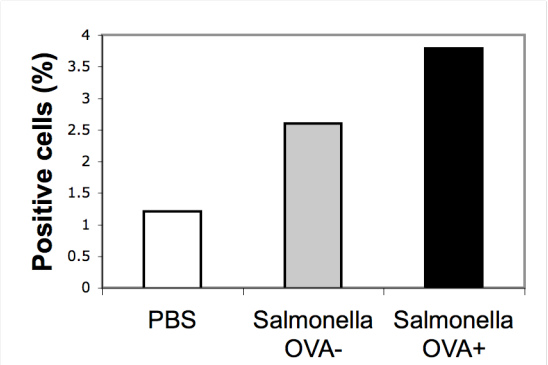

Fig. 6E. IFN-γ positive cells

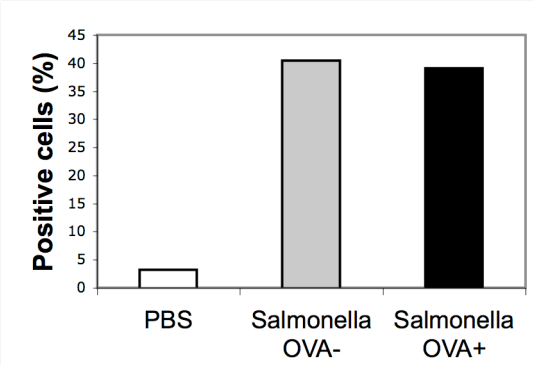

Fig. 6F. IL-2 positive cells

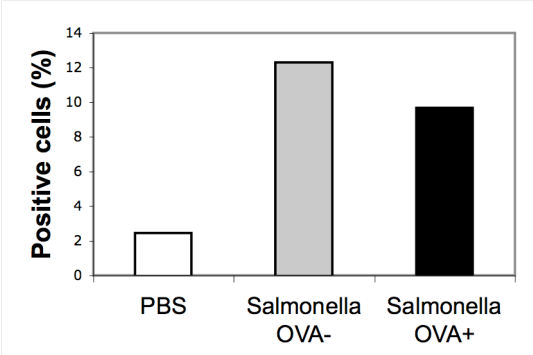

Fig. 6G. IL-4 positive cells

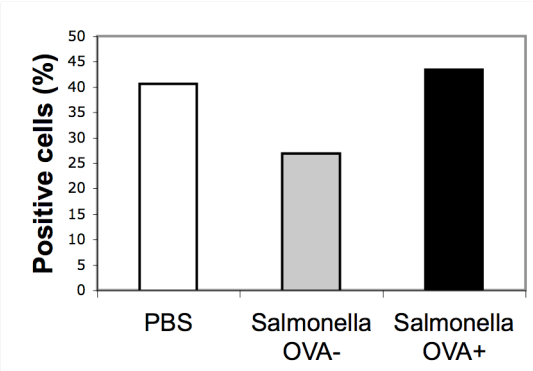

Fig. 6H. FoxP3 positive cells

Figure 6. Flow cytometry of IL-2, IL-4, IFN-γ and FoxP3 positive cells of lymphnodes. Cells were analyzed after challenge at day 28 (prophylactical approach, Fig. 6A-D) and day 70 (therapeutical approach, Fig. 6E-H), respectively. The percentage of IL-2, IL-4, IFN-γ positive cells are referred to the amount of viable cells. FoxP3 positive cells are referred to the amount of $CD4^+$ and $CD25^+$ cells.

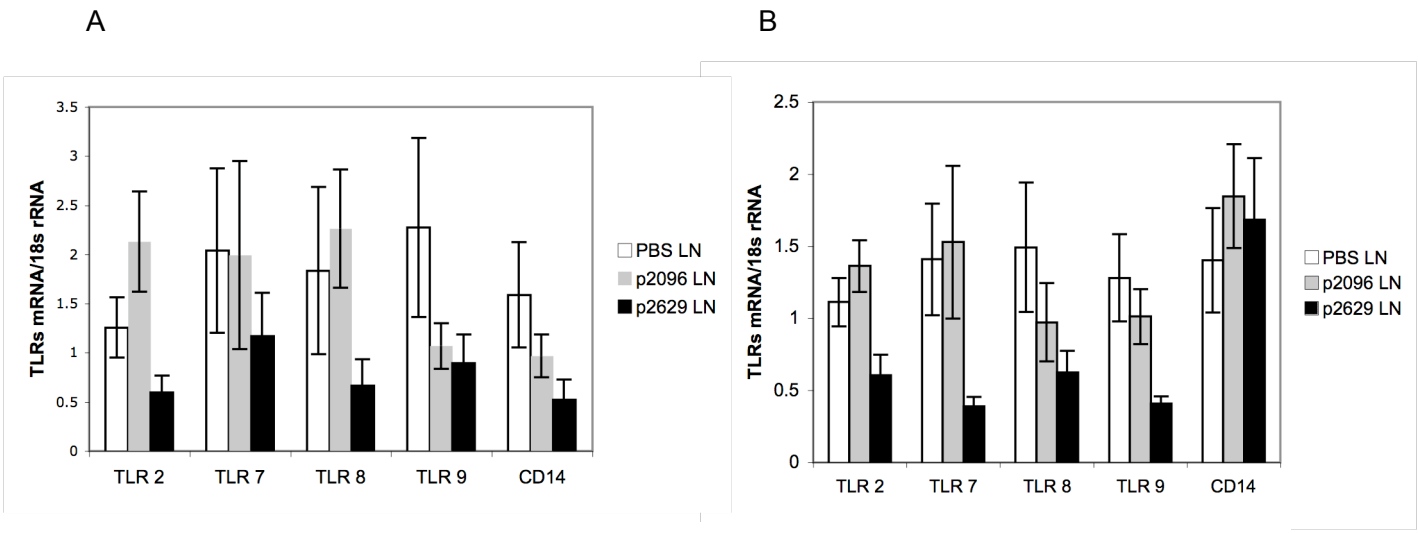

Figure 7. Relative gene expression of different TLRs in lymphnode cells. Expression of mRNA of the indicated TLRs was measured by real-time PCR, RNA yield differences were calculated using 18s RNA as endogenous control and the comparative CT method for relative quantification. Bars represent the mean values for each group; error bars represent standard errors. A: Prophylactical approach; B: Therapeutical approach.

Discussion

Combined intake of microorganisms and low amounts of allergen is a possible method to prevent or treat food allergies. We used *Salmonella enterica* serovar Typhimurium as live carrier for the expression of the model allergen ovalbumin fused on the effector protein sseF. The symptom scores indicated that mice treated with bacteria releasing OVA have less anaphylactic symptoms in a prophylactical as well as a therapeutical approach. These results are further based on elevated IgA levels in feces and decreased expression of Toll-like receptors in lymphnodes. IgA is predominantly found at mucous membrane surfaces and provides an important line of defense against bacteria and viruses, however little is known about the role of IgA in food allergy. Eigenmann et al has recently shown in a similar food allergy model with prophylactic administration of two different attenuated *Salmonella* strains a higher allergen-specific IgA in feces[22] although these bacteria did not express the allergen. Our results show also increased OVA-specific IgA levels in mice treated with not OVA-expressing *Salmonella*, however the titres were clear increased and the anaphylactic symptoms considerably reduced in the group treated with *Salmonella* expressing OVA. It seems that allergen-specific IgA prevents adherence and penetration of OVA and thereby reduces immune inflammatory responses through the mucosal epithelium. Bottcher et al observed in a prospective 2-year study with new-born infants that high levels of secretory IgA were found to be increased in sensitized but clinically tolerant infants suggesting that high levels of secretory IgA may protect against development of allergy[23]. However, pre-treatment of mice with *Bifidobacterium bifidium*, *Lactobacillus acidophilus*, *Bifidobacterium lactis* and *Lactobacillus acidophilus* together with *Bifidobacterium lacti*s before OVA sensitization was

found to lead to a decreased OVA-specific IgA level in fecal samples[24,25]. This indicates that bacteria, particularly the intracellular or extracellular living in the gut influences the IgA response.

The signalling mechanisms of the innate immune system may control susceptibility to allergy. TLRs are pattern recognition receptors which recognize highly conserved microbial structures and thereby initiate and guide the immune responses. In both approaches the expression of different TLRs was decreased in mice treated with *Salmonella* expressing OVA. We presume that the repeated bacterial stimulation developes oral tolerance and may lead to less inflammatory response, what may reduce the anaphylactic symptoms.

Linked to the use of attenuated *Salmonella* strains, different studies described an increased Th1 activity, as indicated by IFN-γ production from antigen-stimulated T cells, high amount of antigen-specific IgG2a levels and decreased antigen-specific IgG1 levels in serum[22,26-29]. Our data are not completely consistent of that since we measured only in the therapeutical approach more IFN-γ positive cells in mice treated with *Salmonella* expressing OVA and the levels of allergen-specific IgG1 and IgG2a/b in serum behave the same trend. These differences may based on the used mouse strain C3H/HeJ which bears a spontaneous mutation in the *tlr4* gene. TLR4 is the receptor for lipopolysaccharid (LPS)[30] which is the main component in the outer membrane of Gram-negative bacteria, such as *Salmonella* and mediates the expression of pro-inflammatory cytokines, such as TNF-a and IL12[31,32] and the induction of adaptive immune response. Through this mutation the induction of adaptive immune response and the production of IgG may be disordered. Furthermore, Husseiny et al[18] compared the humoral immune response against OVA in mice, immunized with the conventional and extensively used auxotrophic *aroA* or the *sseC* carrier strain deficient in SPI2 gene. Both strains were modulated to express OVA either constitutive or regulated. They observed a pronounced increase in OVA-specific IgG titers after immunization with the sseC strain, especially if the OVA gene expression was regulated. This indicates that the bacteria strain as well the type of promoter which regulates the antigen expression influence the immune response. Further analysis with other mouse and bacteria strains are still needed to understand the underlying immunological mechanisms. However our results provide an indication that the use of *Salmonella*, modified genetically to express an allergen, may be a suitable tool to prevent and treat food induced allergy.

Contribution to this work

In vitro experiments were designed, performed, and analyzed by myself at the lab of R. Lauener at the Childrens's Hospital Zürich. I wrote the licenses for animal testing and I planned and coordinate the *in vivo* experiments at the Institute for Labortierkunde (University of Zürich). Animal procedures were supported by Nathalie Frei, Remo Frei and Susanne

Loeliger. Antibody measurements and cytometric analysis were performed by Nathalie Frei and myself.
The manuscript was written by myself.

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CHAPTER B3

Discussion and outlook

In 1948 first adverse reactions to corn, wheat, milk, egg and other frequently ingested food were reported[1]. The prevalence of food allergies among children and adults appears to be rising in Western countries, and there are indication of an increasing prevalence of new food allergy, such as kiwi allergy[2] and sesame seed allergy[3]. The most observed allergic reactions are against egg and milk and certain food allergies seems to be specific for geographic regions, e.g. bird nest soup allergy in Singapore, royal jelly allergy in Hong Kong, and mustard allergy in France[4]. The reasons for food allergy are not defined, however genetic and environmental exposition factors are involved in the development and new therapeutic and prophylactical mechanisms beside avoidance of allergens are needed. Desensitization is a well established method against insect sting allergies or hay fever and there are a growing number of oral immunotherapy studies, in which a food allergen is ingested in gradually increasing amounts over month. The success rates mainly for egg and milk allergy vary and the procedures are not without side effects, including systemic allergic reactions[5-7].

In our considerations concerning the application of desensitization in food allergies, the crucial question was how the release of food allergens occurs on-site where the contact to immune cells is given, primarily in the gut-associated lymphoid tissue (GALT) which is constantly exposed to a variety of antigens and must induce a state of nonresponsiveness (mucosal tolerance) or in case of pathogen invasion elicit a strong T- and B-cell response. The choice to use *Salmonella enterica* serovar Typhimurium as living vectors offered two convincing advantages: First, these bacteria prefentially enter M cells in the gut, which transport them to the lymphoid cells (T and B) in the underlying Peyer`s patches and second, they are studied intensively for vaccination strategies, therefore attenuated strains are investigated and available. The use of attenuated *Salmonella typhimurium*, modified to deliver the allergen OVA had shown in fact a beneficial effect against OVA-induced food allergy in a prophylactical as well as in a therapeutical approach. Therefore, these results suggest that there is presumably a possibility to treat food allergy with the method of desensitization. However, similar to other strategies (modified protein vaccine, plasmid DNA encoded vaccines, sublingual/oral immunotherapy etc.) the use of *Salmonella* typhimurium in food allergy shows still limitations which have to be discussed and resolved.
Although we have seen reduced anaphylactic symptoms in mice treated with *Salmonella* expressing OVA the underlying immunological mechanism is still unclear. Reduced expression of receptors of the innate immunity and elevated levels of $CD4^+CD25^+FOXP3^+$ cells in groups of mice which show less anaphylactic symptoms provides an indication of oral tolerance but further detailed work in this field is necessary. Another phenomenon which is already described in literature and partly observed in *in vitro* analysis by ourselves concerns the combination of attenuation, allergen coding (chromosomal or by plasmid) and allergen

regulation (constitutive or intracellular active). Although a chromosomal gene integration is a stable variant to maintain the gene, the lower amounts of allergen due to the single gene copy is disadvantageous[8]. We observed a stronger allergen expression by the constitutive promoter, however the use of intracellular activated promoters was described to stimulate the immune system more effectively[9-11] compared to the constitutive one. The optimal interplay between gene regulation and location has to be further studied in *in vitro* as well as *in vivo* models. Also the kind of attenuation regarding to safety needs additional investigations, namely the guarantee for local bacterial occurrence in the gut and the exclusion of bacterial persistence.

Finally, it should be considered if this method is suitable to use for other food allergies, especially peanut and/or tree nut allergies, which affect over 1% of Americans and are responsible for a disproportionate number of deaths[12]. In theory, gene exchange of the allergen should be possible, however it is known that peanut allergy is at least in part genetically determined. This form of allergy is about 7-fold more likely to occur in a child with parents or a sibling who is peanut allergic compared to the general population risk[13] and in case of monozygotic twins the likelihood for peanut allergy is 64% if the twin sibling is affected[14]. However, since specific genes have not been identified, it is difficult to estimate if the genetic predisposition countervails the attempt of desensitization. In view of our results of the PASTURE-Study (Chapter A1-A3) a therapy in children with genetic predisposition to food allergy should probably be made early in life when the immune system and the immune tolerance is developed.

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Abbreviations

AICDA	activation-induced cytidine deaminase
ALEX	ALlergy and EndotoXin
APC	antigen presenting cell
AT	Austria
BIR	baculovirus inhibitor domain
CARD	caspase recruitment domain
cDNA	complementary DNA
CB	cord blood
CD	cluster of differentiation
CH	Switzerland
CI	confidence interval
CpG	cytosin-phosphatidyl-guanosin
CIITA	class II transactivator
CT	cholera toxin
Ct	threshold cycle
DC	dendritic cell
DE	Germany
ERK	extracellular signal-regulated kinase
FOXP3	forkhead box P3
FI	Finland
FR	France
Ig	immunoglobulin
IL	interleukin
ILT	immunoglobulin-like transcripts
IKK	inhibitor of nuclear factor-B (IB)-kinase complex
IRAK	IL-1R-associated kinase
IRF	interferon-regulatory factor
ITAM	immunoreceptor tyrosine-based activation motifs
JNK	c-Jun N-terminal kinase
LBP	LPS-binding protein
LPS	lipopolysaccaride
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MHC	major histocompatibility complex
mRNA	messenger ribonucleic acid
MyD88	myeloid differentiation primary-response protein 88

NEMO	NF-κB essential modifier
NF-κB	nuclear factor-κB
NLR	Nod-like receptor
OD	optical density
OVA	ovalbumin
PAMP	pathogen-associated molecular pattern
PARSIFAL	Prevention of Allergy Risk factors for Sensitization in children related to Farming and Anthroposophic Lifestyle
PASTURE	Protection against Allergy: Study in Rural Environments
PI3K	Phosphoinositide 3-kinase
P/I	Phorbol 12-myristate 13-acetate (PMA) /Ionomycin
PMA	Phorbol 12-myristate 13-acetate
PRR	pattern recognition receptor
PYD	pyrin domain
SARM	steril α and armadillo motifs
SIGIRR	Single Ig IL-1–related receptor
SOCS	suppressor of cytokine signalling
SPI	*Salmonella* pathogenicity islands
T3SS	type III secretion system
TCR	T-cell receptor
Th	T-helper cell
TIR	Toll/IL-1R homology
TIRAP/MAL	TIR-associated protein
TLR	Toll-like receptor
TNF	tumor necrosis factor
TOLLIP	Toll-interacting protein
TRAF	TNFR-associated factor
TRAILR	TNF-related apoptosis-inducing ligand receptor
TRAM	TRIF-related adaptor molecule
Treg	T regulatory cell
TREM	triggering receptors expressed by myeloid cells
TRIF/TICAM1	TIR-domain-containing adaptor protein-inducing IFN-β

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Curriculum vitae

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Education

1987-2000	Freie Georgenschule Reutlingen, Germany
2000	Allgemeine Hochschulreife (Abitur)
2000-2002	Studies of Biology, University of Tübingen, Germany
2002-2005	Studies of Microbiology at the Swiss Federal Institute of Technology (ETH), and University of Zurich, Switzerland
2005	Diploma thesis (supervised by PD Dr. Roger Lauener; Prof. Dr. Beat Keller): "Herstellung und Austesten eines löslichen CD14 Fusionsproteins und der Einfluss von Endotoxin auf die Proteinexpression" (Production and testing of a soluble CD14 fusion protein and the influence of endotoxin on protein expression).
2005-2010	PhD thesis in the field of allergy and innate immunity at the University Children′ Hospital, supervised by PD Dr. Roger P. Lauener and Prof. Dr. Beat Keller.

Professional education

2006	Course of animal experiments, Zurich University
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2007	Course in biostatistics, Zurich University
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Publications and manuscripts

- Gerhold K, Avagyan A, Seib C, Frei R, Steinle J, Ahrens B, Dittrich AM, Blumchen K, Lauener R, Hamelmann E. *Prenatal initiation of endotoxin airway exposure prevents subsequent allergen-induced sensitization and airway inflammation in mice*. J Allergy Clin Immunol. 2006 Sep;118(3):666-73

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Congresses (active participation)

- International Disaster and Risk Conference (IDRC), Davos, 2008: Two poster presentations
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